Evidence for progenitor-derivative speciation in sexually deceptive orchids

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Abstract: BACKGROUND AND AIMS: Sexually deceptive orchids of the genus Ophrys use mimicry of pollinator females to attract specific pollinators. Pollinator shifts may drive speciation in Ophrys, since novel pollinators may in principle act as isolating factors immediately. It is thus possible that evolution of novel species occurs rapidly and with a progenitor-derivative pattern. The aims of this study are to compare genetic structure and diversity among widespread and geographically restricted Ophrys taxa, to test whether genetic structure is associated with specific pollinators, and to investigate whether any widespread species may have acted as a progenitor for the evolution of more restricted taxa. METHODS: Genetic differentiation and diversity were investigated in O. leucadica and O. cinereophila, the two taxa of the Ophrys fusca sensu lato complex widespread in the Aegean, and three geographically restricted taxa from Rhodes, O. attaviria, O. parvula and O. persephonae, all differing in their specific pollinators. This was done using amplified fragment length polymorphism (AFLP) DNA fingerprinting, and sequencing of the low-copy nuclear gene LEAFY (LFY). KEY RESULTS: All taxa were found to be separate genetic entities, with O. leucadica forming two geographic groups from the west and east of the Aegean. Genetic structure was significantly shaped by pollinators and geography, and comparison of sequence and AFLP data revealed ancestral polymorphisms shared among several taxa. Among the sampled taxa, O. leucadica harbours the greatest genetic differentiation and geographic structure, and the highest genetic diversity. Part of the genome of O. parvula, endemic to Rhodes, may be derived from O. leucadica. CONCLUSIONS: Pollinators probably influence the genetic structure of the investigated Ophrys species. The genetic pattern identified is consistent with O. leucadica being the oldest of the sampled taxa, making O. leucadica a candidate progenitor species from which more restricted taxa such as O. parvula may have evolved.

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Evidence for progenitor–derivative speciation in sexually deceptive orchids

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• *Background and Aims* Sexually deceptive orchids of the genus *Ophrys* use mimicry of pollinator females to attract specific pollinators. Pollinator shifts may drive speciation in *Ophrys*, since novel pollinators may in principle act as isolating factors immediately. It is thus possible that evolution of novel species occurs rapidly and with a progenitor–derivative pattern. The aims of this study are to compare genetic structure and diversity among widespread and geographically restricted *Ophrys* taxa, to test whether genetic structure is associated with specific pollinators, and to investigate whether any widespread species may have acted as a progenitor for the evolution of more restricted taxa.

• *Methods* Genetic differentiation and diversity were investigated in *O. leucadica* and *O. cinereophila*, the two taxa of the *Ophrys fusca sensu lato* complex widespread in the Aegean, and three geographically restricted taxa from Rhodes, *O. attaviria*, *O. parvula* and *O. persephonae*, all differing in their specific pollinators. This was done using amplified fragment length polymorphism (AFLP) DNA fingerprinting, and sequencing of the low-copy nuclear gene *LEAFY* (*LFY*).

• *Key Results* All taxa were found to be separate genetic entities, with *O. leucadica* forming two geographic groups from the west and east of the Aegean. Genetic structure was significantly shaped by pollinators and geography, and comparison of sequence and AFLP data revealed ancestral polymorphisms shared among several taxa. Among the sampled taxa, *O. leucadica* harbours the greatest genetic differentiation and geographic structure, and the highest genetic diversity. Part of the genome of *O. parvula*, endemic to Rhodes, may be derived from *O. leucadica*.

• *Conclusions* Pollinators probably influence the genetic structure of the investigated *Ophrys* species. The genetic pattern identified is consistent with *O. leucadica* being the oldest of the sampled taxa, making *O. leucadica* a candidate progenitor species from which more restricted taxa such as *O. parvula* may have evolved.

Key words: AFLP, genetic diversity, genetic structure, low-copy nuclear genes, *Ophrys*, pollination, progenitor–derivative speciation, sexually deceptive orchids.

INTRODUCTION

Many orchids are characterized by a high specificity of pollination (Schiestl and Schlüter, 2009). In particular, sexually deceptive orchids, the flowers of which mimic female bees to attract males as pollinators, can attain pollinator-mediated reproductive isolation by differential attraction of pollinator species (Paulus and Gack, 1990; Schiestl and Ayasse, 2002; Peakall *et al.*, 2010; Ayasse *et al.*, 2011; Gaskett, 2011). *Ophrys* is a European and Mediterranean genus of Orchidaceae that is pollinated using a mechanism of sexual deception (Kullenberg, 1961; Paulus and Gack, 1990; Paulus, 2006). *Ophrys* does not offer any reward or incentive for generalized pollinators, and species of this genus are predominantly characterized by pollination by one (or few) specific insect species (Paulus and Gack, 1990; Paulus, 2006). *Ophrys* flowers attract male pollinators by mimicry of key traits of their females and induce pollinator males to mate with the flower, resulting in pollen transfer. The most important trait mimicked by *Ophrys* flowers is the insect virgin female's sex pheromone (Schiestl *et al.*, 1999, 2000), which elicits copulatory behaviour in males and explains the high specificity of pollinator attraction observed in *Ophrys* (Ayasse *et al.*, 2001; Vereecken, 2009). Accordingly, strong floral isolation among co-flowering *Ophrys* species has been reported, whereas post-zygotic mating barriers appear to be largely absent (Ehrendorfer, 1980; Cozzolino *et al.*, 2004; Scopece *et al.*, 2007; Schlüter *et al.*, 2009; Xu *et al.*, 2011; but see Gögler *et al.*, 2009).

In plant species with a high specificity of pollination, such as *Ophrys*, pollinator behaviour can serve as a pre-mating reproductive barrier. In this case, pollinator-mediated reproductive isolation is consistent with ecological speciation (Schluter and Conte, 2009) due to divergent selection on odour phenotypes (see Mant *et al.*, 2005*b*). As long as divergent selection acts on a small number of traits, this is expected to be a 'genic' speciation process (Wu, 2001; Wu and Ting, 2004), in which species differences are initially caused by only a few genes that are the targets of divergent selection, whereas gene flow is effective throughout the majority of the

genome. Such a scenario is especially likely in *Ophrys* because a small number of genes are expected to be responsible for differences in pollinator attraction among species (Schlüter and Schiestl, 2008; Schlüter *et al.*, 2009, 2011), and strong floral isolation, linked to only a few genes of large effect, make rapid speciation by pollinator shift appear likely. Since speciation brought about by a pollinator shift within one population would not be expected to have any impact on pollinator specificity in other populations of the source species, one would expect speciation to follow a progenitor–derivative pattern (see, for example, Levin, 1993; Rieseberg and Brouillet, 1994; Gottlieb, 2003; Levin, 2004; Waser and Campbell, 2004; Crawford, 2010), in which a derivative species arises as a genetic sub-set of the progenitor species without affecting the progenitor. The derivative species should then (*a*) be monophyletic and genetically closely related to the progenitor (which might be paraphyletic); but (*b*) contain only a sub-set of the progenitor's genetic diversity, less genetic population structure and fewer private alleles (Perron *et al.*, 2000). Moreover, (*c*) due to its local origin, the derivative species should be geographically restricted when compared with its more widespread progenitor. Furthermore, (*d*) in the case of *Ophrys*, progenitor and derivative should be isolated by their different pollinators. Therefore, a common *Ophrys* species may give rise to a number of local endemics that are genetically similar to the gene pool from which they are derived. The potential for rapid speciation implies that many *Ophrys* species may be of recent origin, making it difficult to obtain reliable phylogenetic hypotheses. This may be further complicated by the expectation of paraphyly for any species that acted as a progenitor for other species (e.g. Rieseberg and Brouillet, 1994). In practice, many markers commonly used to infer phylogenies do not harbour sufficient variation to obtain a well-supported estimate of relationships within *Ophrys* (Soliva *et al.*, 2001; Bateman *et al.*, 2003; Bernardos *et al.*, 2005; Schlüter *et al.*, 2007*a*; Devey *et al.*, 2008). Nonetheless, *Ophrys* sect. *Pseudophrys* is well supported as a monophyletic group based on molecular data (Soliva *et al.*, 2001; Bateman *et al.*, 2003; Bernardos *et al.*, 2005; Devey *et al.*, 2008). Morphologically, this section is characterized by the direction of the trichomes on the labellum. This determines the orientation of male pollinators on the lip (Ågren *et al.*, 1984; Pirstinger, 1996; Pirstinger and Paulus, 1996), which results in the attachment of pollinaria to an insect's abdomen rather than its head. Within section *Pseudophrys*, *O. fusca senus lato* (*s.l.*) represents the most diverse species complex, relationships within which are poorly understood (but see Schlüter *et al.*, 2007*a*).

The *O. fusca s.l.* group has a pan-Mediterranean distribution, containing a few widely distributed taxa and a large number of highly restricted or endemic taxa. In the Aegean, *O. leucadica* and *O. cinereophila* are two common members of the *O. fusca s.l.* group. *Ophrys leucadica* occurs throughout the Aegean, with the exception of Crete, and may be conspecific with *O. bilunulata* from the west Mediterranean, based upon morphology and pollination biology (Paulus, 2001*b*; Paulus and Salkowski, 2007). *Ophrys cinereophila* is distributed throughout the Aegean, but does not occur in the west Mediterranean (Delforge, 2006). *Ophrys attaviria*, *O. parvula* and *O. persephonae* have much more restricted distributions, restricted to or centred around the east Aegean island of Rhodes (Paulus, 2001*a*; Kreutz, 2003; Paulus and Schlüter, 2007). All five study species co-occur on Rhodes, and differ in their pollinators (Supplementary Data Table S1, available online) and flower labellum size (which is correlated with pollinator body size; Paulus, 2006), but partially overlap in their flowering times (Kretzschmar *et al.*, 2001; Paulus, 2001*a*; Paulus and Schlüter, 2007). The study species are exclusively pollinated by *Andrena* bees, all of which appear to share a common pheromone chemistry (Ayasse *et al.*, 2011). Mimicry of *Andrena* pheromones by other *Ophrys* species has a genic basis (Schlüter *et al.*, 2011) and results in strong pollinator-mediated reproductive isolation (Xu *et al.*, 2011). It is noted that *Ophrys* species-level taxonomy is contended (compare, for example, Delforge, 2006; Pedersen and Faurholdt, 2007), the present study following the taxonomy of Paulus (2001*a*, *b*).

The pattern of widespread vs. restricted taxa may be indicative of the presence of a few progenitor species that gave rise to a number of derivative species. Specifically, *O. leucadica* or *O. cinereophila* may have acted as progenitors for any of the restricted species *O. attaviria*, *O. persephonae* or *O. parvula*. To evaluate this hypothesis, it is necessary to uncover the genetic structure in both the more widely distributed and more restricted *Ophrys* taxa. Here, amplified fragment length polymorphism (AFLP) markers (Vos *et al.*, 1995) were used for this purpose. AFLP data were complemented with sequence data from the putatively single-copy gene *LEAFY* (*LFY*) (Montieri *et al.*, 2004; Schlüter *et al.*, 2007*a*) from *Ophrys* sect. *Pseudophrys* members. Using multiple lines of evidence from pollinator specificity, phylogenetic and population genetic data, the present study seeks to (*a*) elucidate the genetic structure and diversity of *O. leucadica* and *O. cinereophila* in the Aegean, and of restricted taxa from Rhodes; (*b*) test whether genetic structure is associated with pollinators or geography; (*c*) investigate the relationships among taxa; and (*d*) test whether any of the widespread taxa may have acted as a progenitor for the more restricted taxa found on Rhodes.

MATERIALS AND METHODS

Plant material, DNA extraction and fluorescent AFLP reactions

Plant material (Fig. 1, Table 1) was collected in the field and stored in silica gel. Where possible, plant individuals were photographed and representative vouchers deposited in the herbarium at Vienna University (WU), Austria. Populations were sampled so as to address questions at the taxon rather than at the within-population level (for sample sizes, see Table 1). Plants from all populations included in AFLP analyses were tested for specific pollinator attraction in the field, as described previously (Schlüter *et al.*, 2009). Most of the sampled populations were small, with <50 individuals observed. DNA was extracted using a DNeasy plant mini kit (Qiagen, Vienna, Austria) and the manufacturer's protocol. The AFLP analyses followed the procedure of Vos *et al.* (1995), with modifications as detailed in Schlüter *et al.* (2007*b*). Six primer combinations of 5′-fluorophore-labelled *Eco*RI primers and unlabelled *Mse*I primers were used: *Mse*I-CTCG with *Eco*RI-ACT (6-FAM), -ATC (HEX) and -ACC (NED), and *Mse*I-CTAG with

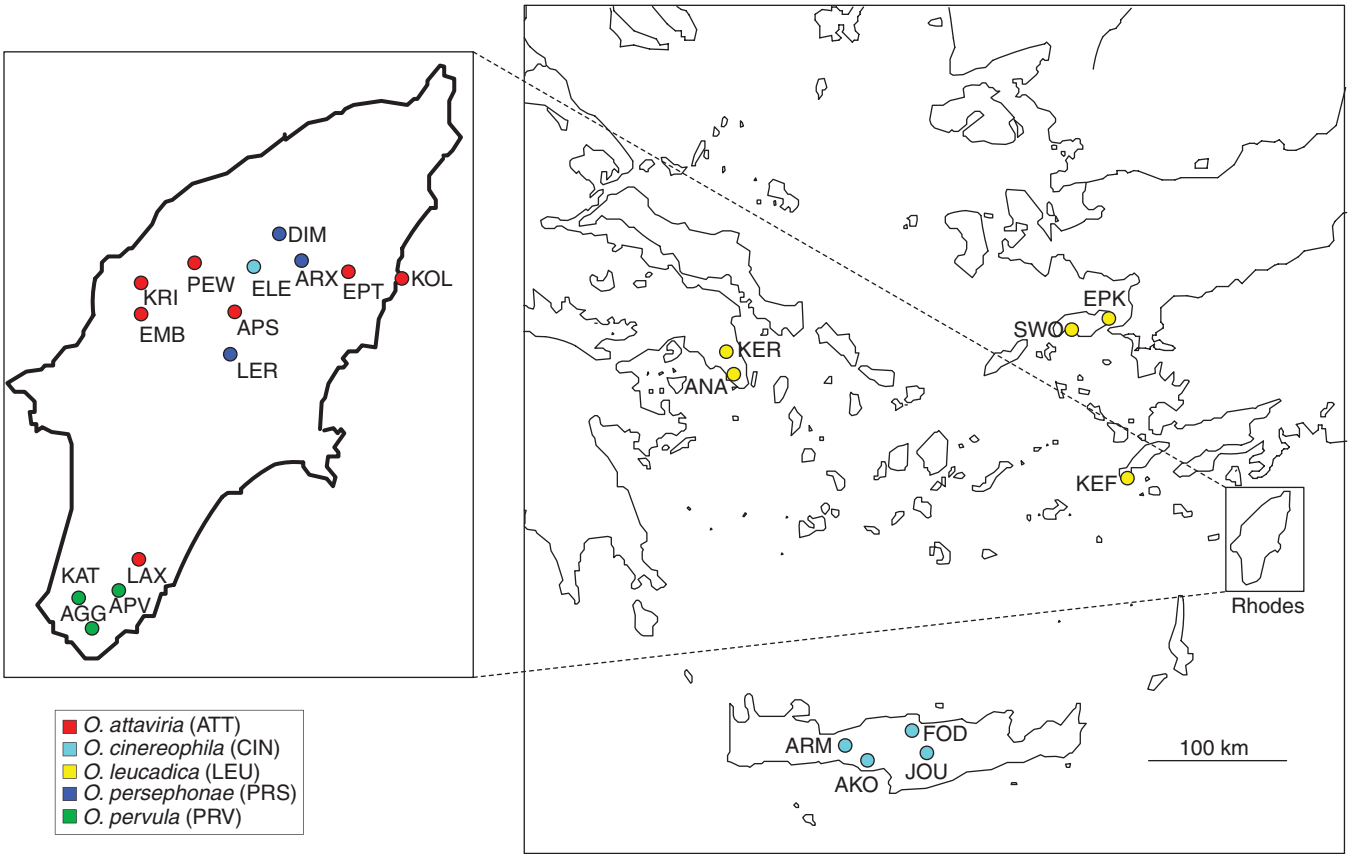


FIG. 1. Map of the Aegean indicating localities from which population samples were taken for AFLP analysis. *Ophrys bilunulata* was sampled from the west Mediterranean and is therefore not indicated on this map. Localities for different taxa are highlighted in different colours, as indicated in the figure. The inset shows details of the island Rhodes. Sampling localities conform to the three-letter codes shown in Table 1.

*Eco*RI-ACT (6-FAM), -AGG (HEX) and -AGC (NED). Genescan-500-ROX (Applied Biosystems, Vienna, Austria) was used as an internal size standard, and AFLP reactions, including appropriate negative and positive controls, were run on a 4 % denaturing polyacrylamide gel in an ABI Prism 377 DNA sequencer (Perkin Elmer Applied Biosystems, Vienna, Austria).

Scoring and data analysis

The AFLP banding patterns were scored manually using Genographer software v.1·6·0 (Benham *et al.*, 1999) as a visual aid. The data set was scored twice independently, coding bands as presence (1) or absence (0), explicitly scoring ambiguous bands as missing data (*?*). Both scorings were first analysed separately, and secondly as a combined data set. AFLP error rates were estimated as suggested by Bonin *et al.* (2004); the mean genotyping error rate among controls and the mean error rate among scorings (of the same fragments) were estimated applying (*a*) strict and (*b*) relaxed criteria. Strict error rates treated *1*/*?* and *0*/*?* band comparisons as errors, whereas these combinations were not treated as erroneous under relaxed criteria.

Maximum-likelihood-based reallocation tests were performed in AFLPOP (Duchesne and Bernatchez, 2002) to test if sampled individuals belonged to their respective putative source populations. Bayesian analysis of population structure was carried out using BAPS 3·2 (Corander *et al.*, 2003), with AFLP data treated as diploid and coding the second allele at every locus as missing data. Clustering of individuals and admixture analysis were performed with the maximum number of populations set to ten. Structure 2·3·1 (Falush *et al.*, 2007) analyses were carried out using the admixture model with correlated allele frequencies, and AFLP data input as diploid, dominant data. Each analysis was run for 100 000 generations, discounting the first 50 % as a burn-in. Analyses were performed in triplicate for $K = 2$ to $K = 10$, and the optimal K value was determined using the method of Evanno *et al.* (2005). Pairwise distance matrices were calculated from AFLP data using an average Jaccard coefficient taking into account missing data, and subjected to principal coordinate analysis (PCoA) in FAMD 1·29 (Schlüter and Harris, 2006). Counting of private bands was done in the same software.

Allele frequencies were estimated with the Bayesian method of Zhivotovsky (1999) in FAMD, using the non-uniform prior from among-population variation, and pairwise population distances calculated using the chord distance (Cavalli-Sforza and Edwards, 1967) in the multilocus formulation of Takezaki and Nei (1996). The chord distance was shown to outperform other population distance methods in recovering the true topology in simulations (Takezaki and Nei, 1996). Pairwise Φ_{ST} values

TABLE 1. *Plant samples used for AFLP analysis, where* n *is the number of individuals sampled from a population*

Code	Region	Locality	*n*	Date	Collector	Population number
O. attaviria D. Rückbrodt & Wenker (*ATT*), *n* = 25						
APS	Rhodes	S. of Apollona	5	25·04·2003	PMS	144
EMB	Rhodes	Embonas	3	24·04·2003	PMS	139
KRI	Rhodes	Kritinia	3	24·04·2003	PMS	137
KOL	Rhodes	Kolymbia	1	23·04·2003	PMS	134
PEW	Rhodes	Profitis Elias (W side)	3	22·04·2003	PMS	126
EPT	Rhodes	Epta Piges	4	20·04·2003	PMS	117
LAX	Rhodes	Lachania	6	23·04·2003	PMS	136
O. bilunulata Risso (BIL), *n* = 7						
CLD	Malaga	Coin Las Delicias	7	09·04·2004	HFP	198
O. cinereophila Paulus & Gack (*CIN*), *n* = 24						
AKO	Crete	Akoumia	3	02·04·2003	HFP	114
ARM	Crete	Armeni	2	02·04·2003	HFP	112
ELE	Rhodes	W. of Eleoussa	8	22·04·2003	PMS	130
FOD	Crete	Fodele	5	01·04·2003	HFP	110
JOU	Crete	Jouchtas	6	30·03·2003	HFP	103
O. leucadica Renz (*LEU*), *n* = 25						
ANA	Attica	Anavissos	5	26·03·2004	M. Fiedler	209
EPK	Samos	Paleokastro	7	21·02·2004	HFP	172
KEF	Kos	Kefalos	3	01·03·2002	HFP	067
KER	Attica	Keratea	2	26·03·2004	M. Fiedler	210
SWO	Samos	Ormos	8	24·02·2004	HFP	184
O. parvula Paulus (*PRV*), *n* = 7						
AGG	Rhodes	Agios Georgios	3	22·04·2003	PMS	131
APV	Rhodes	Agios Pavlos	2	23·04·2003	PMS	135
KAT	Rhodes	Katavia	2	28·03·2004	M. Fiedler	215
O. persephonae Paulus (*PRS*), *n* = 9						
ARX	Rhodes	Archipolis	4	20·04·2003	PMS	118
DIM	Rhodes	Dimylia	3	20·04·2003	PMS	119
LER	Rhodes	Laerma	2	25·04·2003	PMS	142

were calculated in Arlequin 3·0 (Excoffier *et al.*, 2005) and FAMD based on Euclidean and average Jaccard distances. Chord and Φ_{ST} population distances were subjected to UPGMA analysis in FAMD with 1000 bootstrap replicates. Geographic distances among sampling localities were based on the great circle distance via the haversine formula (Sinnott, 1984). A generalized linear model (GLM) with Gaussian error distribution was used to model genetic (pairwise chord and Φ_{ST}) population distance with the explanatory variables geographic distance (log-transformed), shared pollinator (categorical variable), and an interaction term among the two. This analysis was performed in R 2·11·0 (R Development Core Team, 2010).

Analysis of molecular variance (AMOVA) using three levels of hierarchy (taxon, island/region and population) was performed on the AFLP data from *O. cinereophila* and *O. leucadica* using Arlequin 3·0, both across all loci and on a locus-by-locus basis. Similarly, AMOVA using two levels of hierarchy (taxon and population) was calculated for all taxa, and for population groups within *O. leucadica*. Shannon's diversity index (calculated as $H_{Sh} = -\Sigma p_i \log_2 p_i$ where p_i is the frequency of band presence in a locus) and its variance were estimated by bootstrapping using 10 000 pseudo-replicates in FAMD (Schlüter and Harris, 2006), sampling seven randomly chosen individuals per species and iteration, and randomly replacing missing data by 50 % band absences and 50 % band presences. Significance tests for Shannon's index were carried out as suggested previously (Hutcheson, 1970; Magurran, 1988).

DNA sequences

After screening several loci (Schlüter *et al.*, 2007*a*), sequences from the low-copy nuclear gene *LFY* were obtained from members of *Ophrys* sect. *Pseudophrys* to complement the AFLP analysis. Sampling included mostly Aegean taxa of the section, with several accessions of *O. cinereophila* and *O. leucadica* (Supplementary Data Table S2). Several samples (*O. attaviria* 117A; *O. bilunulata* 198A; *O. cinereophila* 114A, 130D; *O. leucadica* 67A, 172A, 209B; *O. persephonae* 119B) were present in both AFLP and sequence data sets, and further samples represented different plant individuals from the same populations (*O. cinereophila* 25A; *O. parvula* 131C) or nearby populations (*O. leucadica* 333A). The nuclear gene *LFY* is probably a single-copy gene in *Ophrys* (Montieri *et al.*, 2004; Schlüter *et al.*, 2007*a*). The 5' fragment of *LFY* was amplified, sequenced and alleles compiled as described previously (Schlüter *et al.*, 2007*a*). The sequenced region covers exon 1, intron 1 and part of exon 2 of *LFY* and is approx. 3 kb in length, of which the intron constitutes two-thirds.

Sequence analysis

The *LFY* sequences were added to the alignment of Schlüter *et al.* (2007*a*) and manually aligned using BioEdit 7·0·1 (Hall, 1999). Since *LFY* sequences were amplified from a presumably nuclear locus, and as such are expected to undergo recombination, recombination among sequences was tested using the

program RDP3 with its default parameters (Martin *et al.*, 2010), either with all sequences in the alignment or with automatic masking of sequences. Three data sets were compiled: (*a*) all observed sequences; (*b*) only non-recombined sequences; and (*c*) all sequences, splitting each recombined sequence into two sequences at the inferred recombination breakpoints and treating the remaining nucleotides as missing data. The phylogenetic relationships among sequences were inferred using MrBayes 3·1·2 (Ronquist and Huelsenbeck, 2003), as detailed in the Supplementary Methods available online.

Observed heterozygosity (H_O) for each species was calculated as the number of *LFY* heterozygotes divided by the total number of individuals sampled for that species. Expected heterozygosity was not calculated because our data do not allow us to estimate allele frequency.

RESULTS

AFLP results

Amplified fragment length polymorphism bands were scored twice for 97 individuals. After removal of bands that occurred only in single individuals, two scorings of the same 655 AFLP markers were available. Since initial analysis suggested congruent results, these two scorings were combined into a single data matrix containing 2·83 % missing data. The mean genotyping error between controls and error rate among scorings (Bonin *et al.*, 2004) were 5·11 % (relaxed) to 7·57 % (strict) and 4·47 % (relaxed) to 7·88 % (strict), respectively.

Principal coordinate analysis of pairwise distances (Fig. 2A), Bayesian clustering and Structure 2·3·1 analyses (both in Fig. 2B) were largely concordant, suggesting seven genetic groups in our data corresponding to the investigated taxa, with the exception of *O. leucadica* which appeared as two groups. One group contained the two western populations from Attica and one the eastern populations from Samos and Kos. Hereafter, these two groups are referred to as eastern (E) or western (W) groups of *O. leucadica.* Two individuals (139A and 184F) were considered to be outliers and excluded from all population-based analyses. Structure analysis suggested the *O. parvula* genome to be admixed (Fig. 2B). At the optimal value of $K = 6$, *O. parvula* is inferred to have genomic contributions of *O. attaviria* and *O. leucadica* group E; only the contribution of *O. leucadica* E is inferred at $K > 6$.

Clustering at the population level using the chord distance supported the above analyses, with *O. parvula* inferred as sister group to the eastern *O. leucadica* populations (Supplementary Data Fig. S1, available online). A GLM revealed that both geographic distance and shared pollinators significantly explain genetic distances (Supplementary Data Table S3), genetic distances being smaller among population pairs with the same pollinator (Supplementary Data Fig. S2). In most GLM analyses, shared pollinators were a more significant factor than geography.

The number of private AFLP bands was highest for *O. leucadica* (Supplementary Data Table S4). Likewise, Shannon's diversity index (Fig. 3; Supplementary Data Table S4) was highest for *O. leucadica*, being significantly greater than that of any other sampled taxon (all $P < 0{\cdot}01$). *Ophrys cinereophila* displayed the second highest Shannon's index (significantly greater than that of the remaining Aegean taxa; all $P < 0{\cdot}01$). Analysis of molecular variance-derived Φ_{ST} values for the taxa studied (Supplementary Data Table S4) generally suggested low intra-taxon differentiation, although this was not the case for *O. leucadica*. Nested AMOVA for *O. leucadica* and *O. cinereophila* (Supplementary Data Table S5), which were both sampled from different geographic regions, showed that both taxa harbour a similar amount of genotypic variation within geographic groups. However, differentiation among geographic groups was much stronger in *O. leucadica*. Inclusion of *O. bilunulata* as an additional group within *O. leucadica* did not alter the above findings (Supplementary Data Tables S4, S5).

Sequence results

A total of 87 *LFY* alleles from 66 *Ophrys* individuals were analysed, of which ten sequences were putatively recombined (Supplementary Data Table S6). For no individual could more than two alleles be found, which is consistent with the hypothesis that the studied taxa are diploids. The same two alleles were found in both *O. sphegodes* 392A and *O. archipelagi* 393A. *LFY* gene genealogies obtained with different treatments of recombined sequences were largely concordant (Fig. 4, Supplementary Data Figs S3, S4). Three groups of sequences were identified from *Ophrys* sect. *Pseudophrys* taxa (Fig. 4, Supplementary Data Table S7): group A contained *O. fusca s.l.* endemics from Crete such as *O. creticola* or *O. creberrima*; group B contained, for example, *O. omegaifera s.l.* and *O. iricolor s.l.*; and group C contained, for example, *O. bilunulata* and *O. lutea s.l.* The allele from *O. parvula* was placed in group A, whereas *O. attaviria* and *O. persephonae* alleles were in group B. Only alleles from *O. leucadica*, *O. cinereophila* and *O. thriptiensis* (endemic to Crete) were found in more than one sequence group. Recombination among *O. fusca s.l.* sequences was found only among sequence groups A and B (Fig. 4, Supplementary Data Table S6). No geographic pattern was discernible for *O. cinereophila* and *O. leucadica LFY* alleles. *Ophrys cinereophila* from Crete had alleles from groups A and B; *O. leucadica* from western localities also used for AFLP (and population KRP nearby) had alleles in groups A and B, while those from eastern localities had alleles in groups A and C, the C alleles being very similar to alleles from *O. bilunulata*. Observed heterozygosity was highest for *O. thriptiensis*, *O. leucadica* and *O. cinereophila* (Supplementary Data Table S7).

DISCUSSION

Genetic structure is shaped by pollinators and geography

Pollinators of *Ophrys* are thought to be highly specific and would be expected to act as isolating factors (Paulus and Gack, 1990), although the effectiveness of pollinators in maintaining species boundaries and preventing hybridization or gene flow among *Ophrys* taxa has been questioned by some authors (e.g. Devey *et al.*, 2008). Phylogenetic studies of *Ophrys* show limited divergence among taxa in nuclear ribosomal ITS (internal transcribed spacer) and chloroplast DNA

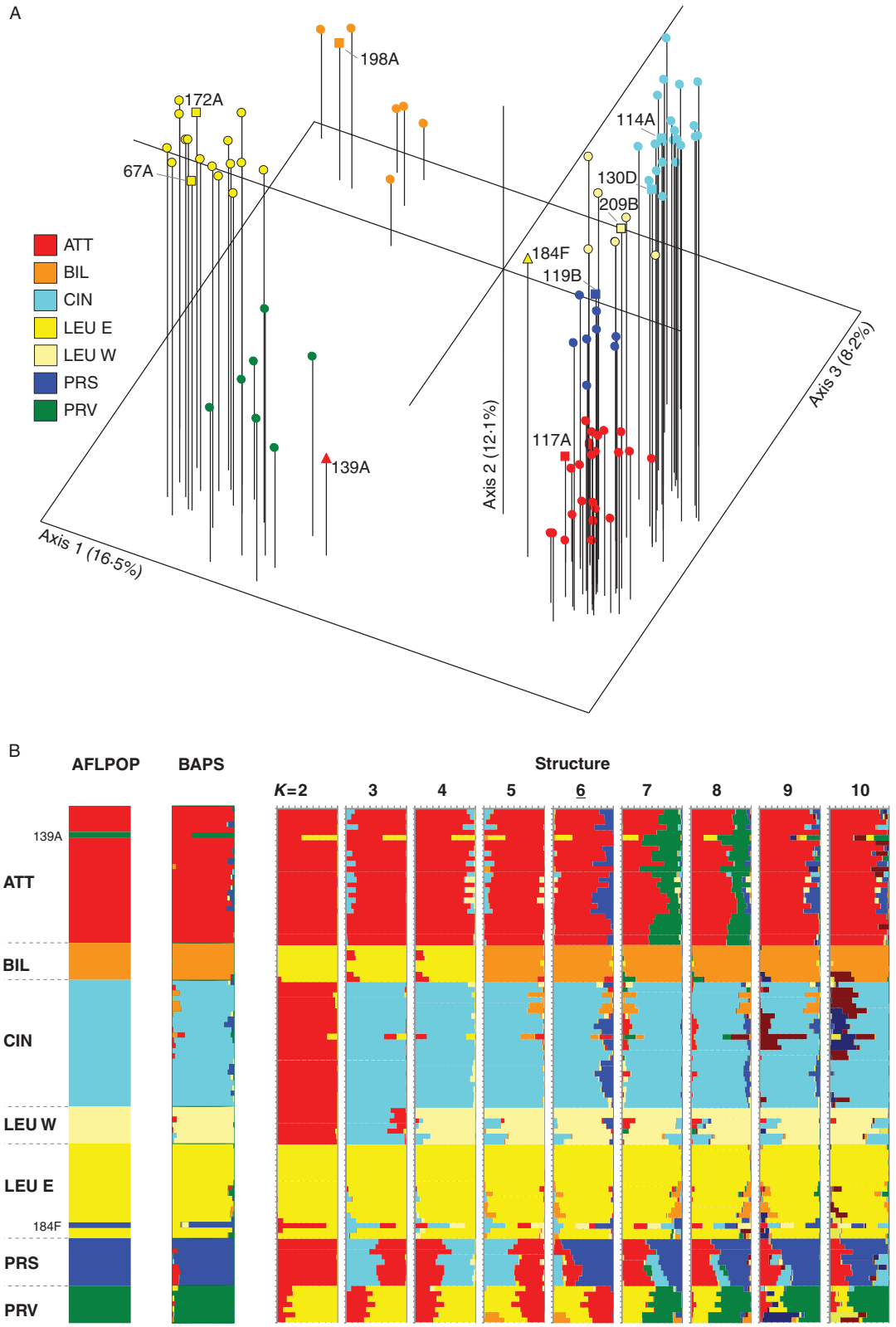


FIG. 2. Results from individual-based analyses of AFLP data. (A) PCoA plot based on an average Jaccard's coefficient after 100 random draws from the interval of possible values (Schlüter and Harris, 2006). Points of different colour represent individuals of different taxa, as indicated in the figure. In the case of *O. leucadica*, two shades of yellow are used to differentiate the two geographical groups of individuals from the east and west of the sampled area, denoted by the letters E and W, respectively. Circles represent samples only present in the AFLP data sets, whereas squares represent samples present in AFLP and sequence data sets (annotated with the sample number). Triangles represent the two individuals treated as outliers. (B) Left to right, results from AFLPOP, BAPS and Structure ($K = 2–10$) analyses, the underlined K value (6) indicating the most likely value following Evanno *et al.* (2005). Species codes are as shown in Table 1.

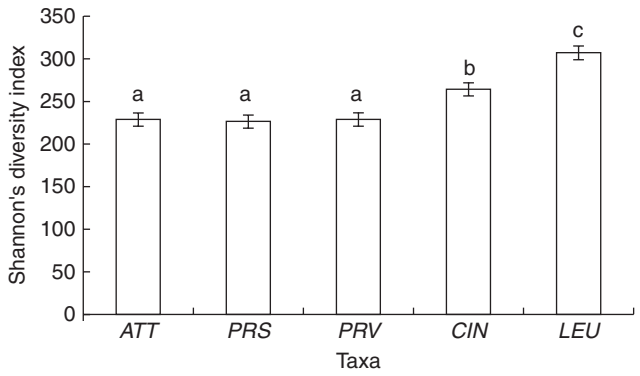


FIG. 3. Shannon's diversity index, a measure of genetic diversity, for the Aegean taxa studied, randomly sampling seven individuals per taxon. The abbreviations for taxa are given in Table 1. Error bars indicate ± s.d. Different letters indicate values that are significantly different from each other ($P < 0{\cdot}01$).

(e.g. Soliva *et al.*, 2001; Bateman *et al.*, 2003; Devey *et al.*, 2008), which can be taken as evidence for fast divergence and radiation of taxa, or as evidence for gene flow and hybridization (or both). Also, previous population studies using molecular markers have yielded conflicting data, some suggesting a weak or absent differentiation among taxa with different pollinators (e.g. Soliva and Widmer, 2003; Mant *et al.*, 2005*b*; Schlüter *et al.*, 2007*b*), whereas in other studies genetic groups delimited by different pollinators were evident (e.g. Grünanger *et al.*, 1998; Caporali *et al.*, 2001; Schlüter *et al.*, 2007*b*; Gögler *et al.*, 2009; Stökl *et al.*, 2009). In this study, we found that apart from *O. leucadica*/*O. bilunulata*, all investigated *Ophrys* taxa were identified as cohesive genetic groups in our AFLP analysis. Since these orchid taxa differ in their specific pollinators (different species of the genus *Andrena*; Supplementary Data Table S1), this supports the role of pollinators in maintaining species boundaries among the taxa analysed. However, the correlation of geographic and genetic population distances in our data set implies that isolation by distance (i.e. drift) cannot be rejected. Genetic drift has recently been highlighted as an important factor in orchid population biology (Tremblay *et al.*, 2005) and could in principle explain the patterns of population structure in presumably neutral AFLP markers that were observed in this study. However, large effective population sizes have been estimated for *Ophrys* (Soliva and Widmer, 2003; Mant *et al.*, 2005*b*), which should mitigate genetic drift. Nonetheless, it is evident that pollinators also have a strong and significant effect on population structure (Supplementary Data Fig. S2, Table S3). In fact, in many analyses, pollinators are more strongly associated with population structure than is geography (Supplementary Data Table S3). Taken together, this implies that the observed population structure in our study taxa is largely (but not entirely) shaped by pollinators.

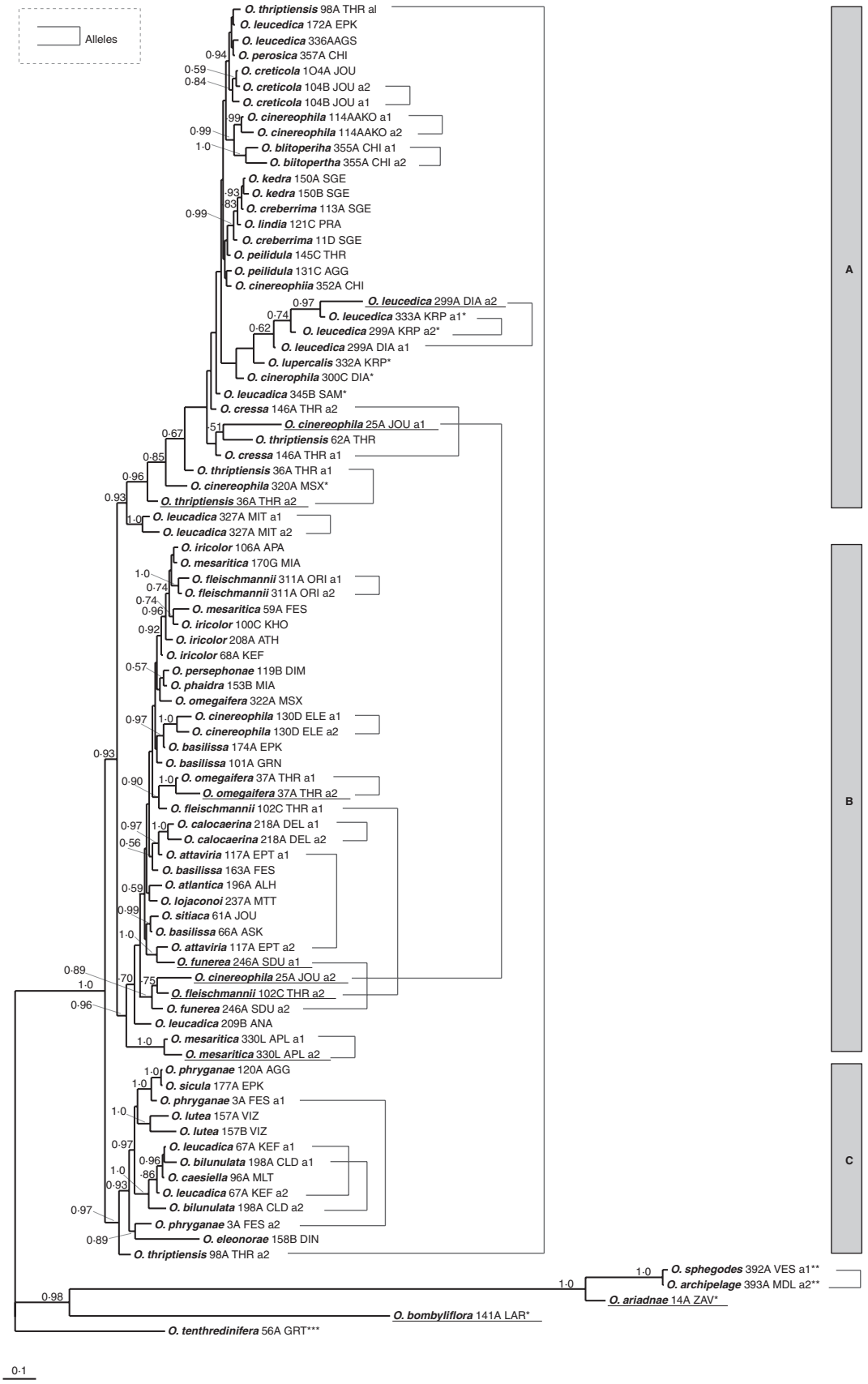
Genetic structure in restricted and widespread taxa

The three taxa sampled only from Rhodes, *O. attaviria*, *O. parvula* and *O. persephonae*, all formed separate groups in AFLP data, which was expected because of their different specific pollinators (Fig. 2; Supplementary Data Table S1). None of these taxa displayed any obvious geographic population structure. In Structure analyses, *O. parvula* showed signs of a mixed genomic composition, which may reflect either ancestral polymorphism or a hybrid origin. A hybrid origin, however, seems unlikely, because this species is genetically separate from the other species with which it shares alleles. Moreover, morphological assessments are not suggestive of hybridity, the flowers (and pollinator) of *O. parvula* being considerably smaller than those of both putative parents, *O. leucadica* and *O. attaviria* (Paulus, 2001*a*; Paulus and Schlüter, 2007).

Strong genetic structure was observed in *O. leucadica*, but not in *O. cinereophila*. This was evident in PCoA (Fig. 2), and in the higher Φ_{ST} value for *O. leucadica* (Supplementary Data Tables S4, S5). The Aegean samples of *O. leucadica* were present in two geographic groups, one from the west Aegean (Attica) and one from the east Aegean (Samos and Kos). If *O. bilunulata* is regarded as conspecific with *O. leucadica* (Paulus, 2001*b*; Paulus and Salkowski, 2007) then the sampled *O. bilunulata* population would represent one further geographic group in *O. leucadica*. In contrast, *O. cinereophila* did not display a similar amount of genetic structure, even though the sample from the Aegean covered a similar geographic range to that from *O. leucadica*. Although in *O. leucadica*, differentiation among geographic groups was higher than in *O. cinereophila* (where samples from Rhodes and Crete were included), both taxa displayed a similar amount of genotypic variation within groups (Supplementary Data Table S5). There are currently no data suggesting a difference in the ecology of *O. leucadica* (and *O. bilunulata*) and *O. cinereophila*, apart from the specialization for different species of pollinating bees. This in turn cannot explain the differences in geographic structure in the two *Ophrys* species, although the apparent absence of the *O. cinereophila*'s pollinator, *Andrena cinereophila*, from the west Mediterranean (Supplementary Data Table S1) (Gusenleitner and Schwarz, 2002) probably explains the absence of this *Ophrys* taxon from that area.

Higher genetic diversity in widespread taxa

The taxa that are common in the Aegean, *O. leucadica* and *O. cinereophila*, displayed significantly greater genetic diversity than the taxa sampled from Rhodes alone (Fig. 3; Supplementary Data Tables S4, S7). This would suggest that the more widespread taxa harbour greater genetic diversity than the more restricted ones. In particular, the highest genetic diversity was found in *O. leucadica*, which is more widely distributed than any of the other sampled taxa, including *O. cinereophila*. This may be expected because more widespread taxa are likely to consist of a higher number of individuals and populations, and therefore have a higher chance for mutations to accumulate. However, while higher genetic diversity (but not population differentiation) is often found in widespread plant species as compared with rare congeners, this is not always the case and cannot be taken as a general trend (Gitzendanner and Soltis, 2000).



Shared ancestral polymorphism

The distinct genetic groups identified from AFLP data contrast markedly with the pattern found among *LFY* alleles, several plant individuals or taxa containing alleles from different sequence groups (Fig. 4; Supplementary Data Table S7). *LFY* was the only marker chosen from a large number of candidates that had enough sequence variation to be informative even within closely related *Ophrys* species (Schlüter *et al.*, 2007*a*). However, the pattern of allelic variation found at this locus implies that phylogenetic reconstructions based on *LFY* sequences may be regarded as gene genealogies representing the evolutionary history at this locus rather than a phylogeny that reflects organismal history. This is further complicated by the inferred presence of recombination at this locus. The observed allelic patterns, in particular the fact that sometimes two alleles found within the same individual were more strongly divergent from each other than from alleles restricted to other species, could be explained by extensive hybridization in the study group or by ancestral polymorphism shared among species (i.e. the retention of allelic diversity that was present prior to speciation in the descendant species). In sexually deceptive systems, genic speciation processes are likely (Schlüter and Schiestl, 2008; Schiestl and Schlüter, 2009; Schlüter *et al.*, 2011), and divergent selection on very few loci may separate incipient species despite ongoing gene flow at other loci in the genome. Initially, species divergence will only be detectable at the few loci under selection. As a consequence, concordant genetic differentiation at multiple neutral loci (like AFLP) will only be detectable later in the process of divergence (see Harrison, 1991). Conversely, an AFLP profile in which species are inseparable (e.g. *O. omegaifera* and *O. sitiaca*) (Schlüter *et al.*, 2007*b*) is consistent with both incomplete divergence and genetic mixing due to hybridization. The fact that the taxa studied here are separable using AFLP implies that the evolutionary history across the entire genome is not obscured by extensive hybridization and thus favours retention of ancestral polymorphism as an explanation for the allelic variation observed at the *LFY* locus. Moreover, hybridization can be rejected as a plausible explanation for the close relationship of *O. bilunulata* and *O. leucadica* alleles (sequence group C), because the respective populations are separated by 2800 km. Therefore, we conclude that incomplete lineage sorting due to retention of ancestral polymorphism is largely responsible for the conflicting patterns among AFLP and *LFY* data.

Ophrys leucadica *as a progenitor species*

The genetic patterns observed in this study are congruent with a scenario in which the widespread *O. leucadica* represents the oldest of the sampled taxa from the *O. fusca s.l.* group and has acted as a progenitor species for more restricted taxa in the Aegean. Whereas the geographic groups found within *O. leucadica* may represent evolutionarily independent lineages (cryptic species) that are convergent in their morphology, phenology and pollinator attraction, there is currently no biological evidence that would strengthen this view, and a greater age of *O. leucadica* seems more plausible given the available data. The hypothesis of a greater age of *O. leucadica* – and therefore a higher likelihood of having acted as a progenitor species – compared with other members of the Aegean *O. fusca* group is in agreement with (*a*) the greater distribution of this species (assuming equal rates of dispersal, habitat and pollinator availability); (*b*) its greater genetic differentiation among populations; (*c*) its greater genetic diversity; and (*d*) the finding of ancestral polymorphism in this species. First, while evolution in *Ophrys* may occur on a short time scale, and highly restricted taxa, such as *O. parvula*, may arise relatively quickly, it is obvious that colonization of the entire Mediterranean basin by *O. leucadica* (if it has the same origin as *O. bilunulata*) or at least the Aegean (if it does not) would require more time. Secondly, *O. leucadica*, being older than the other widespread species, *O. cinereophila*, would explain why geographic groups in *O. leucadica* have 'drifted apart' and show genetic differentiation, whilst such structure is absent from *O. cinereophila*. Thirdly, genetic diversity may likewise be the result of accumulation of mutations over a longer time scale. Conversely, populations (or parts thereof) that diverged from a progenitor species due to selection by a novel pollinator are expected to have reduced genetic diversity because of founder effects. This is consistent with the lower genetic diversities of *O. attaviria*, *O. parvula* and *O. persephonae*, their lower numbers of private AFLP bands, and the observed pattern of *LFY* alleles. Among these taxa, *O. parvula* is inferred to have genomic similarities with *O. leucadica* in Structure analyses, which may reflect an ancestral genomic contribution from the progenitor species.

Although *O. cinereophila* is relatively widespread in the Aegean and is polymorphic for *LFY* alleles, it does not show genetic differentiation among geographic regions. By the same reasoning as above, *O. cinereophila* would be expected to be younger than *O. leucadica* and less likely to have acted as a progenitor for other *O. fusca s.l.* taxa in the Aegean. However, another potential example of a progenitor–derivative species pair may be *O. iricolor* and *O. mesaritica* (also from section *Pseudophrys*), although, in that case, AFLP profiles cannot yet separate the two taxa despite evidence for a pollinator shift and likely strong floral isolation among these species (Schlüter *et al.*, 2009). Therefore, it seems possible that pollinator-mediated progenitor–derivative speciation is common in the genus *Ophrys*.

Progenitor–derivative speciation in sexually deceptive orchids

In his recent review, Crawford (2010) points out that, apart from cases of ecogeographic and mating system differences,

FIG. 4. Relationships among *LFY* alleles from *Ophrys* sect. *Pseudophrys* (and some outgroup individuals) as determined by Bayesian inference phylogeny reconstruction, with brackets indicating alleles (a1 or a2) sampled from the same individual. Plant individuals and accession numbers are listed in Supplementary Data Table S2 (available online). Branches are labelled with Bayesian posterior probabilities (where >0·5) and underlined sequences represent putatively recombined alleles (see Supplementary Data Table S6, available online). Sequence groups A, B and C referred to in the text are indicated by the respective letters. *, partial sequence; **, both alleles were found in *O. sphegodes* 392A and *O. archipelagi* 393A; ***, merged outgroup sequence (see Supplementary Data Table S2).

data on pre-mating barriers to gene flow among progenitor–derivative species pairs are largely lacking, identifying only two such cases in his literature survey: first, *Camassia angusta* (Agavaceae) may be a recent derivative of *C. scilloides*; these species are crossable and a difference in flowering time has been suggested as a reproductive barrier (Ranker and Schnabel, 1986). Secondly, in addition to post-mating barriers, pollinator behaviour has been suggested to act as a partial pre-mating barrier between *Mimulus guttatus* (Phrymaceae) and its derivative *M. nudatus* (Macnair and Gardner, 1998). Hence, *Ophrys* orchids may represent one of the few known cases where specific pollinators are both the main reproductive barrier among progenitor and derivative species, and potentially also the drivers of the speciation process.

Pollinator-mediated progenitor–derivative speciation may, however, be more widespread. For instance, as in *Ophrys*, highly specific pollinators provide the main reproductive barrier among Australian sexually deceptive orchids of the genus *Chiloglottis*, making pollinator-driven speciation due to scent changes likely (Bower and Brown, 2009; Peakall *et al.*, 2010; Ayasse *et al.*, 2011; Gaskett, 2011). This process may have a simple genic basis, allowing for sympatric divergence with gene flow (Mant *et al.*, 2005*a*; Peakall *et al.*, 2010). Thus, the occurrence of progenitor–derivative species patterns in *Chiloglottis* would not be surprising. More generally, one might expect that progenitor–derivative species patterns may be a common result for genic ecological speciation processes, in which strong pre-mating isolation allows the swift establishment of barriers to gene flow in sympatry.

Conclusions

The taxa studied here from the *O. fusca s.l.* group, each with a different pollinator, represent genetically distinct units, pollinators (besides geography) significantly affecting population structure. This supports the role of pollinators in maintaining reproductive isolation among the studied taxa. Secondly, the widespread Aegean taxa *O. leucadica* and *O. cinereophila* have higher genetic diversities than the restricted taxa found on Rhodes, and the comparison of AFLP and sequence data suggests the retention of ancestral polymorphism. Furthermore, *O. leucadica* (whether including *O. bilunulata* or not) shows a strong geographic population structure, which contrasts with *O. cinereophila*. The genetic pattern is consistent with the scenario that *O. leucadica* is a progenitor species for restricted or endemic *Ophrys fusca s.l.* taxa in the Aegean region, in particular *O. parvula*. Genic ecological speciation processes may be expected to result in patterns of progenitor–derivative speciation. This study illustrates that such patterns are indeed detectable in sexually deceptive orchids.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Supplementary methods: details of the phylogenetic sequence analysis. Table S1: summary of the study species and their pollinators. Table S2: plant samples used for sequence analysis. Table S3: generalized linear models of genetic distance, geographic distance and pollinators. Table S4: diversity and differentiation statistics from AFLP data. Table S5: results from nested AMOVA analyses of AFLP data. Table S6: summary of *LFY* recombination analysis. Table S7: summary statistics for *LFY* sequence data. Figure S1: dendrogram of inter-population relationships from AFLP data. Figure S2: analysis of genetic vs. geographic distance between populations. Figure S3: relationships among all non-recombined *LFY* alleles. Figure S4: relationships among all *LFY* alleles and partial alleles.

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LITERATURE CITED

Ågren L, Kullenberg B, Sensenbaugh T. 1984. Congruences in pilosity between three species of *Ophrys* (Orchidaceae) and their hymenopteran pollinators. *Nova Acta Regiae Societatis Scientiarum Upsaliensis* **Ser. V, C, 3**: 15–25.

Ayasse M, Paxton RJ, Tengö J. 2001. Mating behavior and chemical communication in the order Hymenoptera. *Annual Review of Entomology* **46**: 31–78.

Ayasse M, Stökl J, Francke W. 2011. Chemical ecology and pollinator-driven speciation in sexually deceptive orchids. *Phytochemistry* **72**: 1667–1677.

Bateman RM, Hollingsworth PM, Preston J, Yi-Bo L, Pridgeon AM, Chase MW. 2003. Molecular phylogenetics and evolution of Orchidinae and selected Habenariinae (Orchidaceae). *Botanical Journal of the Linnean Society* **142**: 1–40.

Benham JJ, Jeung J-U, Jasieniuk MA, Kanazin V, Blake TK. 1999. Genographer: a graphical tool for automated fluorescent AFLP and microsatellite analysis. *Journal of Agricultural Genomics* **4**: paper 399.

Bernardos S, Crespí A, del Rey F, Amich F. 2005. The section *Pseudophrys* (*Ophrys*, Orchidaceae) in the Iberian Peninsula: a morphometric and molecular analysis. *Botanical Journal of the Linnean Society* **148**: 359–375.

Bonin A, Bellemain E, Bronken Eidesen P, Pompanon F, Brochmann C, Taberlet P. 2004. How to track and assess genotyping errors in population genetic studies. *Molecular Ecology* **13**: 3261–3273.

Bower CC, Brown GR. 2009. Pollinator specificity, cryptic species and geographical patterns in pollinator responses to sexually deceptive orchids in the genus *Chiloglottis*: the *Chiloglottis gunnii* complex. *Australian Journal of Botany* **57**: 37–55.

Caporali E, Grünanger P, Marziani G, Servettaz O, Spada A. 2001. Molecular (RAPD) analysis of some taxa of the *Ophrys bertolonii* aggregate (Orchidaceae). *Israel Journal of Plant Sciences* **49**: 85–89.

Cavalli-Sforza LL, Edwards AWF. 1967. Phylogenetic analysis: models and estimation procedures. *Evolution: International Journal of Organic Evolution* **21**: 550–570.

Corander J, Waldmann P, Sillanpää MJ. 2003. Bayesian analysis of genetic differentiation between populations. *Genetics* **163**: 367–374.

Cozzolino S, D'Emerico S, Widmer A. 2004. Evidence for reproductive isolate selection in Mediterranean orchids: karyotype differences compensate for the lack of pollinator specificity. *Proceedings of the Royal Society of London B: Biological Sciences* **271**: S259–S262.

Crawford DJ. 2010. Progenitor–derivative species pairs and plant speciation. *Taxon* **59**: 1413–1423.

Delforge P. 2006. *Orchids of Europe, North Africa, and the Middle East*, 3rd edn. London: A&C Black.

Devey DS, Bateman RM, Fay MF, Hawkins JA. 2008. Friends or relatives? Phylogenetics and species delimitation in the controversial European orchid genus *Ophrys*. *Annals of Botany* **101**: 385–402.

Duchesne P, Bernatchez L. 2002. AFLPOP: a computer program for simulated and real population allocation, based on AFLP data. *Molecular Ecology Notes* **2**: 380–383.

Ehrendorfer F. 1980. Hybridisierung, Polyploidie und Evolution bei europäisch–mediterranen Orchideen. *Die Orchidee* **Sonderheft**: 15–34.

Evanno G, Regnout S, Goudet J. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology* **14**: 2611–2620.

Excoffier L, Laval G, Schneider S. 2005. Arlequin (version 3·0): an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* **1**: 47–50.

Falush D, Stephens M, Pritchard JR. 2007. Inference of population structure using multilocus genotype data: dominant markers and null alleles. *Molecular Ecology Notes* **7**: 574–578.

Gaskett AC. 2011. Orchid pollination by sexual deception: pollinator perspectives. *Biological Reviews of the Cambridge Philosophical Society* **86**: 33–75.

Gitzendanner MA, Soltis PS. 2000. Patterns of genetic variation in rare and widespread plant congeners. *American Journal of Botany* **87**: 783–792.

Gögler J, Stökl J, Sramkova A, *et al*. 2009. Ménage à trois – two endemic species of deceptive orchids and one pollinator species. *Evolution: International Journal of Organic Evolution* **63**: 2222–2234.

Gottlieb LD. 2003. Rethinking classic examples of recent speciation in plants. *New Phytologist* **161**: 71–82.

Grünanger P, Caporali E, Marziani G, Menguzzato E, Servettaz O. 1998. Molecular (RAPD) analysis on Italian taxa of the *Ophrys bertolonii* aggregate (Orchidaceae). *Plant Systematics and Evolution* **212**: 177–184.

Gusenleitner F, Schwarz M. 2002. Weltweite Checkliste der Bienengattung *Andrena* mit Bemerkungen und Ergänzungen zu paläarktischen Arten (Hymenoptera, Apidae, Andreninae, *Andrena*). *Entomofauna* **Supplement 12**: 1–1280.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**: 95–98.

Harrison RG. 1991. Molecular changes at speciation. *Annual Review of Ecology and Systematics* **22**: 281–308.

Hutcheson K. 1970. A test for comparing diversities based on the Shannon formula. *Journal of Theoretical Biology* **29**: 151–154.

Kretzschmar H, Kretzschmar G, Eccarius W. 2001. *Orchideen auf Rhodos. Ein Feldführer durch die Orchideenflora der 'Insel des Lichts'*. Bad Hersfeld, Germany: Selbstverlag H. Kretzschmar.

Kreutz CAJ. 2003. *Feldführer der türkischen Orchideen*. Landgraaf, Germany: C. A. J. Kreutz, Selbstverlag.

Kullenberg B. 1961. Studies in *Ophrys* pollination. *Zoologiska Bidrag från Uppsala* **34**: 1–340.

Levin DA. 1993. Local speciation in plants: the rule not the exception. *Systematic Botany* **18**: 197–208.

Levin DA. 2004. The ecological transition in speciation. *New Phytologist* **161**: 91–96.

Macnair MR, Gardner M. 1998. The evolution of edaphic endemics. In: Howard DJ, Berlocher SH eds. *Endless forms: species and speciation.* New York: Oxford University Press, 157–171.

Magurran AE. 1988. *Ecological diversity and its measurement*. London: Croom Helm.

Mant J, Bower CC, Weston PH, Peakall R. 2005*a*. Phylogeography of pollinator-specific sexually deceptive *Chiloglottis* taxa (Orchidaceae): evidence for sympatric divergence? *Molecular Ecology* **14**: 3067–3076.

Mant J, Peakall R, Schiestl FP. 2005*b*. Does selection on floral odor promote differentiation among populations and species of the sexually orchid genus *Ophrys*? *Evolution: International Journal of Organic Evolution* **59**: 1449–1463.

Martin DP, Lemey P, Lott M, Moulton V, Posada D, Lefeuvre P. 2010. RDP3: a flexible and fast computer program for analysing recombination. *Bioinformatics* **26**: 2462–2463.

Montieri S, Gaudio L, Aceto S. 2004. Isolation of the *LFY/FLO* homologue in *Orchis italica* and evolutionary analysis in some European orchids. *Gene* **333**: 101–109.

Paulus HF. 2001*a*. Daten zur Bestäubungsbiologie und Systematik der Gattung *Ophrys* in Rhodos (Griechenland) mit Beschreibung von *Ophrys parvula*, *Ophrys persephonae*, *Ophrys lindia*, *Ophrys eptapigiensis* spp. nov. aus der *Ophrys fusca s.str.* Gruppe und *Ophrys cornutula* spec. nov. aus der *Ophrys oestrifera*-Gruppe (Orchidaceae und Insecta, Apoidea). *Berichte aus den Arbeitskreisen Heimische Orchideen* **18**: 38–86.

Paulus HF. 2001*b*. Material zu einer Revision des *Ophrys fusca s.str.* Artenkreises I. *Ophrys nigroaenea-fusca*, *O. colletes-fusca*, *O. flavipes-fusca*, *O. funerea*, *O. forestieri* oder was ist die typische *Ophrys fusca* Link 1799 (Orchidaceae)? *Journal Europäischer Orchideen* **33**: 121–177.

Paulus HF. 2006. Deceived males – pollination biology of the Mediterranean orchid genus *Ophrys* (Orchidaceae). *Journal Europäischer Orchideen* **38**: 303–353.

Paulus HF, Gack C. 1990. Pollinators as prepollinating isolation factors: evolution and speciation in *Ophrys* (Orchidaceae). *Israel Journal of Botany* **39**: 43–79.

Paulus HF, Salkowski H-E. 2007. Bestäubungsbiologische Untersuchungen an Winterorchideen auf der Ägäis-Insel Kos (Orchidaceae und Insecta, Hymenoptera, Apoidea). *Berichte aus den Arbeitskreisen Heimische Orchideen* **24**: 4–30.

Paulus HF, Schlüter PM. 2007. Neues aus Kreta und Rhodos: Bestäubungsbiologie und molekular-genetische Trennung in der *Ophrys fusca* – Gruppe, mit Neubeschreibungen von *Ophrys phaidra* Paulus nov.sp., *O. pallidula* Paulus nov.sp. und *O. kedra* Paulus nov.sp. aus Kreta (Orchidaceae und Insecta, Apoidea). *Jahresberichte des Naturwissenschaftlichen Vereins in Wuppertal* **60**: 101–151.

Peakall R, Ebert D, Poldy J, *et al*. 2010. Pollinator specificity, floral odour chemistry and the phylogeny of Australian sexually deceptive *Chiloglottis* orchids: implications for pollinator-driven speciation. *New Phytologist* **188**: 437–450.

Pedersen HÆ, Faurholdt N. 2007. *Ophrys: the bee orchids of Europe*. Kew, UK: Kew Publishing.

Perron M, Perry DJ, Andalo C, Bousquet J. 2000. Evidence from sequence-tagged-site markers of a recent progenitor-derivative species pair in conifers. *Proceedings of the National Academy of Sciences, USA* **97**: 11331–11336.

Pirstinger PM. 1996. *Untersuchung der Lippenbehaarung der Gattung* Ophrys *(Orchidaceae) und ihrer Bestäuberweibchen (Apoidea).* Mag. rer. nat. thesis, University of Vienna, Vienna, Austria.

Pirstinger PM, Paulus HF. 1996. Congruences in pilosity between species of *Ophrys* (Orchidaceae) and the females of their hymenopteran pollinators (Apoidea). *Proceedings of the XX International Congress of Entomology.* Firenze, Italy.

Ranker TA, Schnabel AF. 1986. Allozymic and morphological evidence for a progenitor–derivative species pair in *Camassia* (Liliaceae). *Systematic Botany* **11**: 433–445.

R Development Core Team. 2010. *R: a language and environment for statistical computing*. 2·11·0 ed. Vienna, Austria: R Foundation for Statistical Computing. http://www.r-project.org/

Rieseberg LH, Brouillet L. 1994. Are many plant species paraphyletic? *Taxon* **43**: 21–32.

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.

Schiestl FP, Ayasse M. 2002. Do changes in floral odor cause speciation in sexually deceptive orchids? *Plant Systematics and Evolution* **234**: 111–119.

Schiestl FP, Schlüter PM. 2009. Floral isolation, specialized pollination, and pollinator behavior in orchids. *Annual Review of Entomology* **54**: 425–446.

Schiestl FP, Ayasse M, Paulus HF, *et al*. 1999. Orchid pollination by sexual swindle. *Nature* **399**: 421–422.

Schiestl FP, Ayasse M, Paulus HF, *et al*. 2000. Sex pheromone mimicry in the early spider orchid (*Ophrys sphegodes*): patterns of hydrocarbons as the key mechanism for pollination by sexual deception. *Journal of Comparative Physiology A* **186**: 567–574.

Schluter D, Conte GL. 2009. Genetics and ecological speciation. *Proceedings of the National Academy of Sciences, USA* **106**: 9955–9962.

Schlüter PM, Harris SA. 2006. Analysis of multilocus fingerprinting data sets containing missing data. *Molecular Ecology Notes* **6**: 569–572.

Schlüter PM, Schiestl FP. 2008. Molecular mechanisms of floral mimicry in orchids. *Trends in Plant Science* **13**: 228–235.

Schlüter PM, Kohl G, Stuessy TF, Paulus HF. 2007*a*. A screen of low-copy nuclear genes reveals the *LFY* gene as phylogenetically informative in closely related species of orchids (*Ophrys*). *Taxon* **56**: 493–504.

Schlüter PM, Ruas PM, Kohl G, Ruas CF, Stuessy TF, Paulus HF. 2007*b*. Reproductive isolation in the Aegean *Ophrys omegaifera* complex (Orchidaceae). *Plant Systematics and Evolution* **267**: 105–119.

Schlüter PM, Ruas PM, Kohl G, Ruas CF, Stuessy TF, Paulus HF. 2009. Genetic patterns and pollination in *Ophrys iricolor* and *O. mesaritica* (Orchidaceae): sympatric evolution by pollinator shift. *Botanical Journal of the Linnean Society* **159**: 583–598.

Schlüter PM, Xu S, Gagliardini V, *et al*. 2011. Stearoyl-acyl carrier protein desaturases are associated with floral isolation in sexually deceptive orchids. *Proceedings of the National Academy of Sciences, USA* **108**: 5696–5701.

Scopece G, Musacchio A, Widmer A, Cozzolino S. 2007. Patterns of reproductive isolation in Mediterranean orchids. *Evolution: International Journal of Organic Evolution* **61**: 2623–2624.

Sinnott RW. 1984. Virtues of the haversine. *Sky and Telescope* **68**: 159.

Soliva M, Widmer A. 2003. Gene flow across species boundaries in sympatric, sexually deceptive *Ophrys* (Orchidaceae) species. *Evolution: International Journal of Organic Evolution* **57**: 2252–2261.

Soliva M, Kocyan A, Widmer A. 2001. Molecular phylogenetics of the sexually deceptive orchid genus *Ophrys* (Orchidaceae) based on nuclear and chloroplast DNA sequences. *Molecular Phylogenetics and Evolution* **20**: 78–88.

Stökl J, Schlüter PM, Stuessy TF, *et al*. 2009. Speciation in sexually deceptive orchids: pollinator-driven selection maintains discrete odour phenotypes in hybridizing species. *Biological Journal of the Linnean Society* **98**: 439–451.

Takezaki N, Nei M. 1996. Genetic distances and reconstruction of phylogenetic trees from microsatellite DNA. *Genetics* **144**: 389–399.

Tremblay RL, Ackerman JD, Zimmerman JK, Calvo RN. 2005. Variation in sexual reproduction in orchids and its evolutionary consequences: a spasmodic journey to diversification. *Biological Journal of the Linnean Society* **84**: 1–54.

Vereecken NJ. 2009. Deceptive behavior in plants. I. Pollination by sexual deception in orchids: a host–parasite perspective. In: Baluska F, ed. *Plant–environment interactions.* Heidelberg: Springer Verlag, 203–222.

Vos P, Hogers R, Bleeker M, *et al*. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**: 4407–4414.

Waser NM, Campbell DR. 2004. Ecological speciation in flowering plants. In: Dieckmann U, Doebeli M, Metz JAJ, eds. *Adaptive speciation.* Cambridge, UK: Cambridge University Press, 264–277.

Wu C-I. 2001. The genic view of the process of speciation. *Journal of Evolutionary Biology* **14**: 851–865.

Wu C-I, Ting C-T. 2004. Genes and speciation. *Nature Reviews Genetics* **5**: 114–122.

Xu S, Schlüter PM, Scopece G, *et al*. 2011. Floral isolation is the main reproductive barrier among closely related sexually deceptive orchids. *Evolution: International Journal of Organic Evolution*: doi:10.1111/j.1558-5646.2011.01323.x.

Zhivotovsky LA. 1999. Estimating population structure in diploids with multilocus dominant DNA markers. *Molecular Ecology* **8**: 903–913.

SUPPLEMENTARY DATA

Methods: phylogenetic sequence analysis

Insertion–deletion (indel) data were coded as binary data using simple indel coding (Simmons and Ochoterena, 2000) in GapCoder software (Young and Healy, 2003). Every sequence data set was divided in 8 partitions (3 partitions for every codon position in the 2 exons, the intron, and the indels), and substitutional models were chosen for each exon and intron using the AIC criterion in MrModelTest 2.3 (Nylander, 2004). Among the observed sequences, exon 1, intron 1, exon 2 and indels represented 442, 2638, 334 and 126 characters, respectively. Indel characters were treated as restriction characters with ascertainment bias set to 'variable', as suggested in the MrBayes 3.1.2 2 (Ronquist and Huelsenbeck, 2003) software manual. Two independent Markov chain Monte Carlo (MCMC) runs were performed for each data set (4 chains each), using default settings, but sampling one tree every 1000 generations. All runs reached apparent stationarity of *log*-likelihood scores and the analysis was performed until convergence of runs was reached (when the average standard deviation of split frequencies between runs fell below 0.01), discarding results prior to reaching stationarity as a burn-in. In the case of the data set with split recombined sequences (containing substantial missing data), analysis chains had not converged after 100 million generations, seemingly due to parameter interactions among tree length, α-shape, proportion of invariable sites, and among-partition rate variation parameters. Hence, in this case, only the run with higher likelihood was analysed, making sure all parameters had high effective sample sizes (all > 1000, except for tree length, which was 535.4).

LITERATURE CITED

Nylander JAA. 2004. MrModeltest v2. Evolutionary Biology Centre, Uppsala University, Uppsala, Sweden, Program distributed by the author.

Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics,* **19**: 1572-1574.

Simmons MP, Ochoterena H. 2000. Gaps as characters in sequence-based phylogenetic analyses. *Systematic Biology,* **49**: 369-381.

Young ND, Healy J. 2003. GapCoder automates the use of indel characters in phylogenetic analysis. *BMC Bioinformatics,* **4**: 6.

SUPPLEMENTARY DATA

TABLE S1. Summary of the study species' distributions, pollinators, and pollinators' distributions, compiled from the literature. Pollinator records (mostly based on pollinator choice experiments), floral morphology and phenology of the studied *Ophrys* species are listed in Paulus (2001*a*) and Paulus and Schlüter (2007). The distributions of pollinators (all from the genus *Andrena*) are taken from Gusenleitner and Schwarz (2002). The sex pheromone of *A. flavipes* and its mimicry by *O. bilunulata* have previously been investigated (Schiestl and Ayasse, 2002).

Orchid	Pollinator	Orchid distribution	Pollinator distribution
O. attaviria	*A. labialis*[a]	Rhodes; E Aegean	Most of Europe; Western N Africa; Turkey
O. cinereophila	*A. cinereophila*	Aegean	Aegean; Greece; Balkans; Turkey; Cyprus; Israel
O. leucadica/*O. bilunulata*	*A. flavipes*	Mediterranean	Most of Europe; Western N Africa; Near East
O. parvula	*A. tomora*[b]	S Rhodes	Sicily; Aegean; Greece; Turkey
O. persephonae	Not determined[c]	Rhodes; SW Turkey	Unknown

[a] Not attractive to *A. cinereophila* or *A. flavipes*; weakly attractive to *A. nigroaenea* (but no removal of pollinia).
[b] Not attractive to *A. flavipes*.
[c] Not attractive to *A. flavipes*, *A. nigroaenea*, *A. tomora*, and *Blitopertha lineolata*.

TABLE S2. Plant samples used for sequence analysis, indicating taxonomic identity, sampling locality and a 3-letter code for the locality. EMBL # indicates the sequence accession numbers in the public sequence database.

Taxon	Locality, Country	Code	Date	Collector	Accession	EMBL #	Ref.[a]
O. archipelagi GÖLZ & REINHARD	Marina di Lesina, Gargano, Italy	MDL	20.03.2007	PMS	393A	FR872578	1
O. ariadnae PAULUS	Zavros, Crete, Greece	ZAV	29.03.2002	PMS	14A	FR872512	1
O. atlantica MUNBY	Alhaurin de la Torre, Spain	ALH	08.04.2004	HFP	196A	AM489434	2
O. attaviria RÜCKBRODT & WENKER	Epta Piges, Rhodes, Greece	EPT	20.04.2003	PMS	117A	FR872532, FR872533	1
O. basilissa ALIBERTIS & REINHARD	Asklepion, Kos, Greece	ASK	27.02.2002	HFP	66A	AM489423	2
O. basilissa ALIBERTIS & REINHARD	Gournia, Crete, Greece	GRN	29.03.2003	HFP	101A	FR872525	1
O. basilissa ALIBERTIS & REINHARD	Phaistos, Crete, Greece	FES	20.02.2004	PMS	163A	FR872547	1
O. basilissa ALIBERTIS & REINHARD	Klima, Samos, Greece	EPK	21.02.2004	HFP	174A	AM489432	2
O. bilunulata RISSO	Coin Las Delicias, Spain	CLD	09.04.2004	HFP	198A	AM489435, FR872550	1, 2
O. blitopertha PAULUS	Chios, Greece	CHI	15.04.2005	HFP	355A	FR872574, FR872575	1
O. bombyliflora LINK	Lardos/Laerma, Rhodes, Greece	LAR	25.04.2003	PMS	141A	FR872539	1
O. caesiella DELFORGE	Malta, Malta	MLT	01.01.2003	HFP	96A	FR872522	1
O. calocaerina DEVILLERS-TERSCHUREN & DEVILLERS	Delphi, Greece	DEL	12.04.2004	MH	218A	FR872552, FR872553	1
O. cinereophila PAULUS & GACK	Jouchtas, Crete, Greece	JOU	03.04.2002	PMS	25A	FR872513, FR872514	1
O. cinereophila PAULUS & GACK	Akoumia, Crete, Greece	AKO	02.04.2003	HFP	114A	AM489427, AM489428	2
O. cinereophila PAULUS & GACK	Eleoussa, Rhodes, Greece	ELE	22.04.2003	PMS	130D	FR872536, FR872537	1
O. cinereophila PAULUS & GACK	Diakofti, Kythira, Greece	DIA	18.03.2005	PMS	300C	FR872559	1
O. cinereophila PAULUS & GACK	Mesochori, Karpathos, Greece	MSX	23.03.2005	PMS	320A	FR872562	1
O. cinereophila PAULUS & GACK	Chios, Greece	CHI	14.04.2005	HFP	352A	FR872573	1
O. creberrima PAULUS	Spili/Gerakari, Crete, Greece	SGE	02.04.2003	HFP	111D	FR872530	1
O. creberrima PAULUS	Spili/Gerakari, Crete, Greece	SGE	02.04.2003	HFP	113A	FR872531	1
O. cressa PAULUS	Thripti, Crete, Greece	THR	04.05.2003	HFP	146A	FR872540, FR872541	1
O. creticola PAULUS	Jouchtas, Crete, Greece	JOU	30.03.2003	HFP	104A, B	AM489426, FR872528, FR872529	1, 2
O. eleonorae DEVILLERS-TERSCHUREN & DEVILLERS	Dingli Cliffs, Malta	DIN	30.12.2003	HFP	158B	FR872546	1
O. fleischmannii HAYEK	Thripti, Crete, Greece	THR	29.03.2003	HFP	102C	FR872526, FR872527	1
O. fleischmannii HAYEK	Orino, Crete, Greece	ORI	20.03.2005	PMS	311A	FR872560, FR872561	1
O. funerea VIVIANI	Sa Duchessa, Sardinia, Italy	SDU	04.04.2004	JS	246A	FR872555, FR872556	1
O. iricolor DESFONTAINES	Kephalos, Kos, Greece	KEF	02.03.2002	HFP	68A	FR872521	1
O. iricolor DESFONTAINES	Kato Horio, Crete, Greece	KHO	29.03.2003	HFP	100C	AM489425	2

O. iricolor DESFONTAINES	Agies Paraskies, Crete, Greece	APA	30.03.2003	HFP	106A	AM489419	2
O. iricolor DESFONTAINES	Athens, Greece	ATH	26.03.2004	MF	208A	AM489436	2
O. kedra PAULUS	Spili/Gerakari, Crete, Greece	SGE	07.05.2003	HFP	150A, B	AM489431, FR872542	1, 2
O. leucadica RENZ	Kephalos, Kos, Greece	KEF	01.03.2002	HFP	67A	AM489424, FR872520	1, 2
O. leucadica RENZ	Palaeokasta, Samos, Greece	EPK	21.02.2004	HFP	172A	FR872549	1
O. leucadica RENZ	Anavissos, Greece	ANA	26.03.2004	MF	209B	FR872551	1
O. leucadica RENZ	Diakofti, Kythira, Greece	DIA	18.03.2005	PMS	299A	FR872557, FR872558	1
O. leucadica RENZ	Mitada, Kythira, Greece	MIT	18.03.2005	PMS	327A	FR872564, FR872565	1
O. leucadica RENZ	Koropi, Greece	KRP	26.03.2005	HFP	333A	FR872569, FR872570	1
O. leucadica RENZ	S of Argostoli, Kephallonia, Greece	AGS	27.03.2005	HFP	336A	FR872571	1
O. leucadica RENZ	Sami, Kephallonia, Greece	SAM	29.03.2005	HFP	345B	FR872572	1
O. lindia PAULUS	Prasonisi, Rhodes, Greece	PRA	21.04.2003	PMS	121C	FR872535	1
O. lojaconoi DELFORGE	Mattinata, Gargano, Italy	MTT	20.04.2004	HFP	237A	FR872554	1
O. lupercalis DEVILLERS-TERSCHUREN & DEVILLERS	Koropi, Greece	KRP	26.03.2005	HFP	332A	FR872568	1
O. lutea CAVANILLES	Viznar, Spain	VIZ	27.04.2003	PS & GMS	157A, B	FR872544, FR872545	1
O. mesaritica PAULUS, ALIBERTIS & ALIBERTIS	Phaistos, Crete, Greece	FES	13.02.2001	HFP	59A	FR872518	1
O. mesaritica PAULUS, ALIBERTIS & ALIBERTIS	Miamou, Crete, Greece	MIA	24.02.2004	PMS	170G	FR872548	1
O. mesaritica PAULUS, ALIBERTIS & ALIBERTIS	Agia Pelagia, Kythira, Greece	APL	17.03.2005	PMS	330L	FR872566, FR872567	1
O. omegaifera FLEISCHMANN	Thripti, Crete, Greece	THR	25.03.2002	HFP	37A	AM489420, FR872517	1, 2
O. omegaifera FLEISCHMANN	Mesochori, Karpathos, Greece	MSX	23.03.2005	PMS	322A	FR872563	1
O. pallidula PAULUS	Thripti, Crete, Greece	THR	04.05.2003	HFP	145C	AM489430	2
O. parosica DELFORGE	Chios, Greece	CHI	15.04.2005	HFP	357A	FR872576	1
O. parvula PAULUS	S of Kattavia, Rhodes, Greece	AGG	22.04.2003	PMS	131C	FR872538	1
O. persephonae PAULUS	Dimilia, Rhodes, Greece	DIM	20.04.2003	PMS	119B	FR872534	1
O. phaidra PAULUS	Miamou, Crete, Greece	MIA	09.05.2003	HFP	153B	FR872543	1
O. phryganae DEVILLERS-TERSCHUREN & DEVILLERS	Phaistos, Crete, Greece	FES	27.03.2002	PMS	3A	FR872510, FR872511	1
O. phryganae DEVILLERS-TERSCHUREN & DEVILLERS	S of Kattavia, Rhodes, Greece	AGG	21.04.2003	PMS	120A	AM489429	2
O. sicula TINEO	Klima, Samos, Greece	EPK	22.02.2004	HFP	177A	AM489433.2	2
O. sitiaca PAULUS, ALIBERTIS & ALIBERTIS	Jouchtas, Crete, Greece	JOU	14.02.2001	HFP	61A	AM489422	2
O. sphegodes MILLER	Vesuvio, Naples, Italy	VES	19.03.2007	PMS	392A	FR872577	1
O. tenthredinifera WILLDENOW	Gourtinia, Crete, Greece	GRT	??.02.2001	HFP	56A	AM489421	2[a]
O. thriptiensis PAULUS	Thripti, Crete, Greece	THR	25.03.2002	HFP	36A	FR872515, FR872516	1
O. thriptiensis PAULUS	Thripti, Crete, Greece	THR	16.02.2001	HFP	62A	FR872519	1
O. thriptiensis PAULUS	Thripti, Crete, Greece	THR	29.03.2003	HFP	98A	FR872523, FR872524	1

Collectors: GMS – G. M. Schneeweiß; HFP – H. F. Paulus; JS – J. Stökl; MF – M. Fiedler; MH – M. Hirth; PMS – P. M. Schlüter; PS – Peter Schönswetter.

References: 1 – this study; 2 – Schlüter *et al*. (2007a).

[a] Sequence from this accession was merged with published *O. tenthredinifera* exon sequences (EMBL accession numbers AB088443 and AB088444 for exon 1 and 2, respectively) from Montieri *et al*. (2004) for the purpose of the analysis, so as to obtain a full-length outgroup sequence from this taxon.

TABLE S3. Generalised linear models. Genetic distance (pairwise chord distance or pairwise $\Phi_{ST,}$ as indicated) was modelled with the explanatory variables 'Geography' (i.e., log-transformed geographic distance), 'Shared Pollinator' (categorical variable), and their interaction term 'Geography × Shared Pollinator'. The analysis was performed on three different geographic scales: 'All Regions' (all populations in the study), 'Only Aegean' (which excluded western Mediterranean *O. bilunulata* populations) and 'Only Rhodes' (which only included populations from Rhodes).

	Chord distance					Φ_{ST}				
	Estimate	s.e.	*t*-value	*P*-value	Sig.[a]	Estimate	s.e.	*t*-value	*P*-value	Sig.[a]
All Regions										
Intercept	0.035622	0.006201	5.745	2.68E-08	**	0.01048	0.037493	0.28	0.78008	
Geography	0.015744	0.001393	11.302	<2.00E-16	**	0.059308	0.008422	7.042	1.84E-11	**
Shared Pollinator	0.044489	0.007627	5.833	1.68E-08	**	0.336286	0.046114	7.292	4.04E-12	**
Geography × Shared Pollinator	-0.00691	0.001627	-4.245	3.09E-05	**	-0.0362	0.009838	-3.68	0.000286	**
Only Aegean										
Intercept	0.044715	0.007098	6.3	1.53E-09	**	0.008351	0.045789	0.182	0.8554	
Geography	0.012763	0.001856	6.877	5.85E-11	**	0.059991	0.011972	5.011	1.09E-06	**
Shared Pollinator	0.04894	0.008589	5.698	3.74E-08	**	0.28461	0.055406	5.137	6.01E-07	**
Geography × Shared Pollinator	-0.00709	0.002095	-3.385	0.000838	**	-0.02432	0.013517	-1.799	0.0734	
Only Rhodes										
Intercept	0.059428	0.006756	8.797	6.72E-15	**	0.124571	0.04676	2.664	0.00868	*
Geography	0.006389	0.002	3.194	0.00175	*	0.009518	0.013845	0.687	0.493	
Shared Pollinator	0.034643	0.008007	4.326	2.97E-05	**	0.185332	0.055422	3.344	0.00108	*
Geography × Shared Pollinator	-0.00158	0.002219	-0.714	0.47666		0.018804	0.015358	1.224	0.223	

[a] Significance (Sig.) is indicated: * $P < 0.01$; ** $P < 0.001$.

TABLE S4. Diversity and differentiation statistics for the Aegean taxa studied, as well as *O. bilunulata*, *O. bilunulata* + *O. leucadica*, and groups within *O. leucadica*. The abbreviations for taxa are the same as given in Table 1 in the main text. H_{Sh} indicates Shannon's diversity index, a measure of genetic diversity. Standard deviations for H_{Sh} values were obtained by bootstrapping (10 000 pseudo-replicates). $H_{Sh,N=7}$ represents Shannon's diversity when only N=7 (equivalent to the sample size of the smallest group analysed) randomly chosen individuals were evaluated during the resampling procedure. Mean Φ_{ST} over all loci (Euclidean distance; all $P < 10^{-4}$) is a measure of genetic differentiation. $N_{private}$ indicates the number of private (unique) AFLP bands found for a group of individuals.

Species/Group	H_{Sh} ± s.d.	$H_{Sh,N=7}$ ± s.d.	Mean Φ_{ST}	$N_{private}$
Aegean taxa				
ATT	261.4 ± 7.1	228.8 ± 7.7	0.102	10
PRS	230.4 ± 7.3	226.4 ± 7.6	0.085	11
PRV	228.6 ± 7.6	228.7 ± 7.8	0.184	8
CIN	303.9 ± 6.9	264.2 ± 7.7	0.111	29
LEU	346.8 ± 7.1	307.1 ± 8.1	0.315	49
Other groups				
BIL	273.0 ± 8.2	273.1 ± 8.2	n/a	32
LEU + BIL	403.9 ± 6.3	368.1 ± 7.7	0.378	106
LEU E	287.1 ± 7.1	238.1 ± 7.6	0.091	24
LEU W	209.5 ± 7.6	209.5 ± 7.5	0.341	18

TABLE S5. Results from nested AMOVA analyses of AFLP data from *O. cinereophila* (CIN), *O. leucadica* (LEU), and *O. leucadica* + *O. bilunulata* (LEU+BIL). The AMOVAs were designed to reflect the following population structure (population codes as in Table 1 in the main text): CIN = (CinEle), (CinAko, CinArm, CinFod, CinJou); LEU = (LeuAna, LeuKer), (LeuEpk, LeuKef, LeuSwo); LEU + BIL = (BilCld), (LeuAna, LeuKer), (LeuEpk, LeuKef, LeuSwo).

	Among regions	Within regions, among pop.	Among populations	Φ_{ST}	Φ_{SC}	Φ_{CT}
CIN	2.9 %	9.1 %	88.0 %	0.120 ***	0.093 ***	0.029 ^{ns}
LEU	34.9 %	9.4 %	55.7 %	0.443 ***	0.145 ***	0.348 *
LEU + BIL	36.5 %	8.2 %	55.3 %	0.447 ***	0.129 ***	0.365 **

Significance: ^{ns} $P \geq 0.1$; * $P < 0.1$; ** $P < 0.05$; *** $P < 10^{-4}$.

TABLE S6. Summary of *LFY* recombination analysis with RDP3. The putatively recombined region of an allele is indicated with reference to the sequence alignment and to the actual sequence (in brackets). Method refers to the different recombination detection methods in RDP3 that revealed recombination when using all sequences in the data set, or after automatic sequence masking (after forward slash). The methods are: G, GENECONV; C, Chimaera; S, SiScan; M, Maxchi; 3, 3Seq, and B, Bootscan. The method with the lowest *P*-value is underlined and the corresponding *P*-value listed in the 'Min *P*' column. Column 'Part' lists the designations of the partial allele sequences (R1 or R2) and 'L' lists the lengths of these partial sequences. In addition, the most similar sequences are listed for each partial allele, as determined by the minimum pairwise distance among sequences (excluding gaps). Where multiple sequences are listed, they are equidistant from the partial allele.

Recombinant sequence	Rec'd Region	Method	Min *P*	Part	L	Most similar sequences
O. ariadnae 14A	933 - 1029	G/G	0.0045	R1	634	*O. archipelagi* 393A; *O. sphegodes* 392A
	(438 - 532)			R2	95	*O. blitopertha* 355A a1 & 355A a2; *O. cinereophila* 25A a1 R1, 114A a1, 114A a2, 300C & 352A; *O. creberrima* 113A; *O. cressa* 146A a1 & 146A a2; *O. creticola* 104A, 104B a1 & 104B a2; *O. funerea* 246A a1 R1; *O. kedra* 150A & 150B; *O. leucadica* 172A, 299A a1, 333A a2, 336A & 345B; *O. lindia* 121C; *O. lupercalis* 332A; *O. mesaritica* 330L a1; *O. pallidula* 145C; *O. parosica* 357A; *O. parvula* 131C; *O. thriptiensis* 36A a1 & 98A a1
O. bombyliflora 141A	2031 - 2326	S/S	0.0018	R1	1414	*O. omegaifera* 37A a2 R1
	(1030 - 1306)			R2	277	*O. omegaifera* 322A
O. cinereophila 25A a1	2097 - 1037	B,M/B,M	0.0006	R1	1638	*O. cressa* 146A a1; *O. leucadica* 345B; *O. lupercalis* 332A; *O. omegaifera* 37A a2 R1
	(1889 - 876)			R2	1014	*O. cinereophila* 25A a2 R1
O. cinereophila 25A a2	1933 - 1115	M,C,3/-	0.0008	R1	1880	*O. phaidra* 153B
	(1733 - 956)			R2	778	*O. cinereophila* 300C; *O. funereal* 246A a1 R1; *O. leucadica* 333A a1, 333A a2 & 345B
O. fleischmannii 102C a2	1441 - 2096	S,3/-	0.0001	R1	1868	*O. phaidra* 153B
	(1256 - 1895)			R2	640	*O. cinereophila* 114A a1; *O. creticola* 104A, 104B a1 & 104B a2; *O. funerea* 246A a1 R1; *O. leucadica* 172A, 299A a1 & 336A; *O. lindia* 121C; *O. lupercalis* 332A; *O. parosica* 357A; *O. parvula* 131C; *O. pallidula* 145C; *O. thriptiensis* 98A a1
O. funerea 246A a1	1791 - 1037	-/M,C,3	0.0067	R1	1899	*O. cinereophila* 25A a2 R2; *O. fleischmannii* 102C a2 R2; *O. leucadica* 345B; *O. lupercalis* 332A
	(1601 - 891)			R2	711	*O. basilissa* 66A
O. leucadica 299A a2	2570 - 2986	G,B,M,C,S,3/	4.0E-14	R1	2460	*O. leucadica* 333A a1
	(2117 - 2256)	G,B,M,C,S,3		R2	140	*O. leucadica* 299A a1
O. mesaritica 330L a2	2150 - 223	-/B,M,S,3	3.0E-05	R1	755	*O. cinereophila* 300C & 320A; *O. leucadica* 333A a1, 333A a2 & 345B; *O. lupercalis* 332A; *O. mesaritica* 330L a1; *O. omegaifera* 322A
	(1950 - 104)			R2	1847	*O. mesaritica* 330L a1
O. omegaifera 37A a2	2624 - 130	S/-	0.0017	R1	280	*O. attaviria* 117A a1 & 117A a2; *O. cinerophila* 25A a1 R1 & 320A; *O. cressa* 146A a1; *O. leucadica* 209B, 333A a1, 333A a2 & 345B; *O. omegaifera* 322A; *O. thriptiensis* 62A & 98A a2
	(2119 - 11)			R2	2109	*O. thriptiensis* 36A a2 R2
O. thriptiensis 36A a2	1852 - 1086	M,3/M,3	2.8E-05	R1	2038	*O. leucadica* 333A a1
	(1717 - 991)			R2	727	*O. omegaifera* 37A a1 & 37A a2 R2

TABLE S7. The number of sampled individuals, alleles and putatively recombined sequences from each species, highlighting those species in bold for which at least three sequences were available. In addition, observed heterozygosity (H_O) and sequence group (A-C; see Fig. 4) are listed for each species. Where one species contained sequences from several groups, the number of sequences in each group is indicated in brackets. Group D (not indicated in Fig. 4) contains outgroup sequences and group X represents either group A or B (assignment unclear).

Species	$N_{individuals}$	$N_{alleles}$	H_O	$N_{recombined}$	Sequence group
O. archipelagi[a]	1	2	1	0	D
O. ariadnae	1	1	0	1	D
O. atlantica	1	1	0	0	B
O. attaviria	1	2	1	0	B
O. basilissa	**4**	**4**	**0**	**0**	**B**
O. bilunulata	1	2	1	0	C
O. blitopertha	1	2	1	0	A
O. bombyliflora	1	1	0	1	D
O. calocaerina	1	2	1	0	B
O. caesiella	1	1	0	0	C
O. cinereophila	**6**	**9**	**0.5**	**2**	**A(6), B(3)**
O. creberrima	2	2	0	0	A
O. cressa	1	2	1	0	A
O. creticola	2	3	0.5	0	A
O. archipelagi	1	2	1	0	D
O. fleischmannii	2	4	1	1	B
O. funereal	1	2	1	1	B
O. iricolor	**4**	**4**	**0**	**0**	**B**
O. eleonorae	1	1	0	0	C
O. kedra	2	2	0	0	A
O. leucadica	**8**	**12**	**0.5**[b]	**1**	**A(7), B(1), C(2), X(2)**
O. lindia	1	1	0	0	A
O. lojaconoi	1	1	0	0	B
O. lupercalis	1	1	0	0	A
O. lutea	2	2	0	0	C
O. mesaritica	**3**	**4**	**0.33**	**1**	**B**
O. omegaifera	2	3	0.5	1	B
O. parosica	1	1	0	0	A
O. pallidula	1	1	0	0	A
O. phaidra	1	1	0	0	B
O. phryganae	2	3	0.5	0	C
O. persephonae	1	1	0	0	B
O. parvula	1	1	0	0	A
O. sicula	1	1	0	0	C
O. sitiaca	1	1	0	0	B
O. sphegodes[a]	1	2	1	0	D
O. tenthredinifera	1	1	0	0	D
O. thriptiensis	**3**	**5**	**0.67**	**1**	**A(4), C(1)**

[a] *O. archipelagi* and *O. sphegodes* alleles were identical.

[b] When *O. bilunulata* is included in *O. leucadica*, $H_O = 0.56$.

SUPPLEMENTARY DATA

FIG. S1. UPGMA dendrogram based on chord distance calculated from population allele frequencies, with bootstrap support indicated. OTUs are labelled with three letters abbreviating the taxon, followed by three letters indicating the population (as presented in Table 1 in the main text). Different colours highlight taxa (left, as in Fig. 1), or genetic groups from PCoA analysis (right, as in Fig. 2), E and W denote *O. leucadica* individuals from the east and west of the sampled area, respectively.

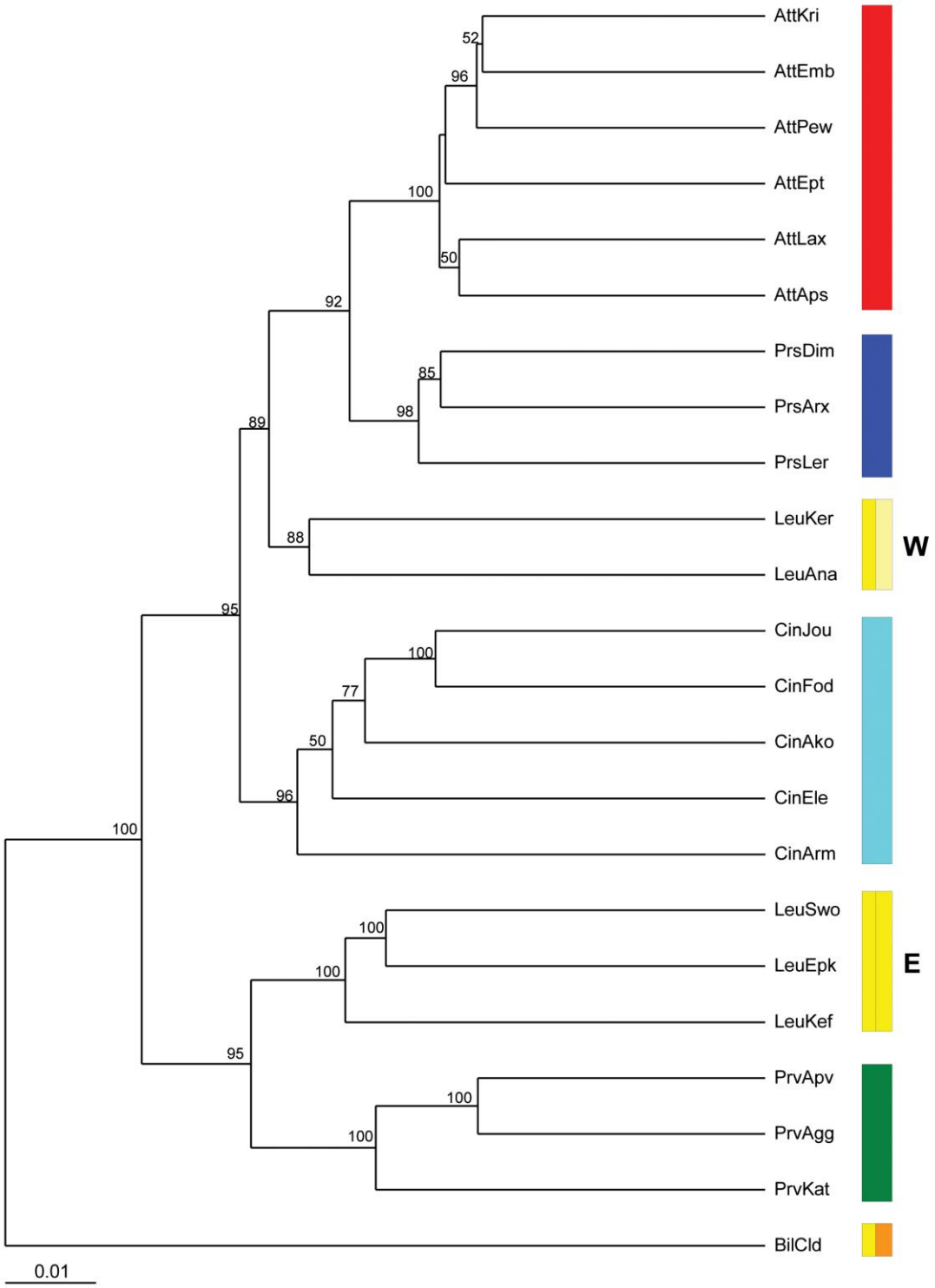

FIG. S2. Analysis of genetic versus geographic distance between populations, for population pairs with the same (blue) or different pollinators (red). (A, B) Correlation among genetic and log-transformed geographic distances. (A, C) Chord distance (shared pollinators: adjusted $R^2 = 0.345$, $P < 10^{-5}$; different pollinators: adjusted $R^2 = 0.747$, $P < 10^{-5}$). (B, D) Pairwise Φ_{ST} (shared pollinators: adjusted $R^2 = 0.403$, $P < 10^{-5}$; different pollinators: adjusted $R^2 = 0.099$, $P < 10^{-5}$). (C, D) Relative density (% of maximum density) of data points for a given genetic distance. The peak inter-population distances are smaller for populations sharing the same pollinator as compared to those with different pollinators.

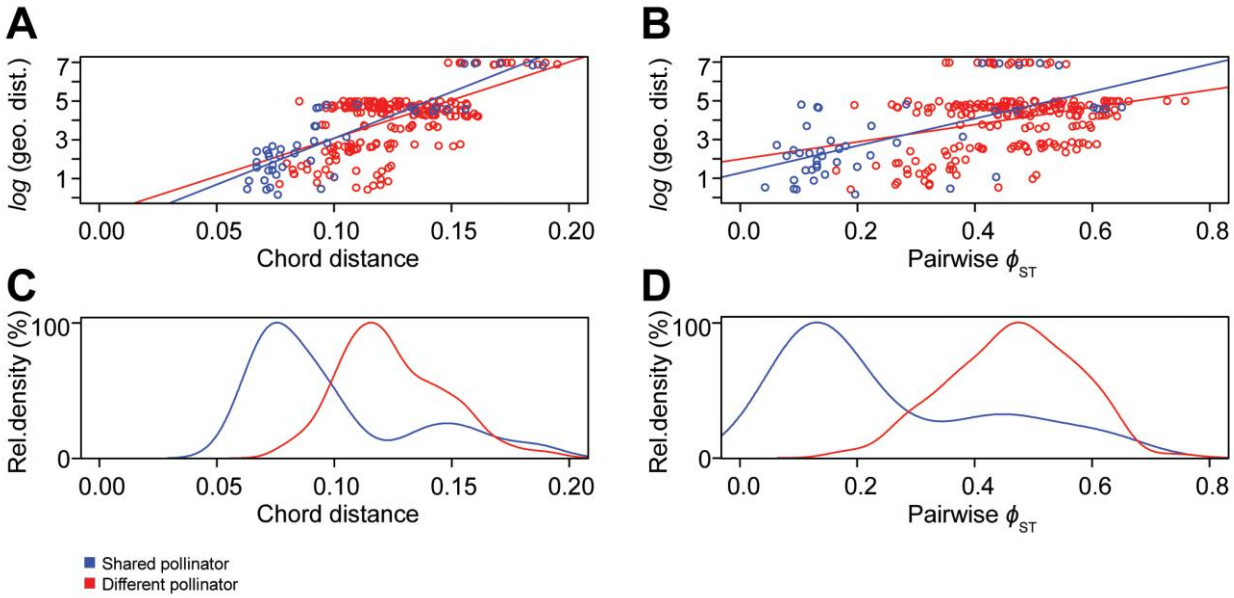

FIG. S3. Relationships among all non-recombined *LFY* alleles from *Ophrys* section *Pseudophrys* as determined by Bayesian inference phylogeny reconstruction, with brackets indicating alleles (a1 or a2) sampled from the same individual. Plant individuals and accession numbers are listed in Table S2. Branches are labelled with Bayesian posterior probabilities (where >0.5). *, partial sequence; **, both alleles were found in *O. sphegodes* 392A and *O. archipelagi* 393A; ***, merged outgroup sequence (see Table S2).

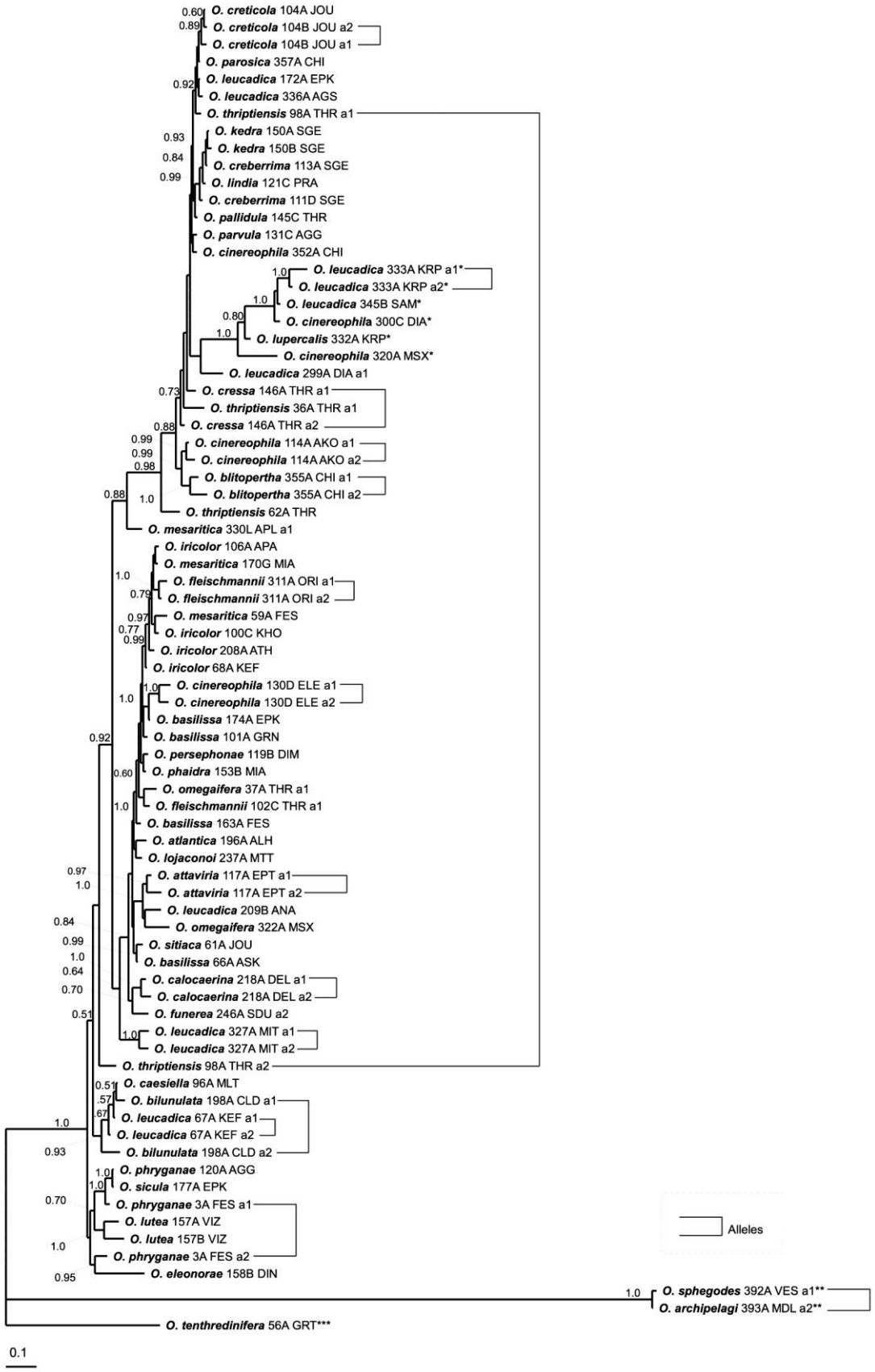

FIG. S4. Relationships among all *LFY* alleles and partial alleles from *Ophrys* section *Pseudophrys* as determined by Bayesian inference phylogeny reconstruction. Alleles are denoted a1 and a2; putatively recombined alleles are further splits into partial alleles R1 and R2 (Table S6). Blue brackets indicate alleles (a1 or a2) sampled from the same individual and red brackets indicate partial recombined fragments of the same allele (R1 or R2). Plant individuals and accession numbers are listed in Table S2. Branches are labelled with Bayesian posterior probabilities (where >0.5). *, partial sequence; **, both alleles were found in *O. sphegodes* 392A and *O. archipelagi* 393A; ***, merged outgroup sequence (see Table S2).